Effects of perfluorooctanesulfonate (PFOS) and perfluorononanoic acid (PFNA) on protein expression and steroidogenesis in the human adrenocortical carcinoma cell line H295R

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Abstract

The main aim of this study was to investigate effects of the perfluorinated compounds perfluorooctanesulphonate (PFOS) and perflouorononanoic acid (PFNA) using the human adrenocortical carcinoma cell line – H295R. Perfluorinated compounds (PFCs) are stable chemicals used widely in products such as paint and stain repellants. These compounds are known to be able to act as endocrine disruptors. H295R is a well-known and a much used model because among other reasons of its ability to produce hormones related to the stereoidogenesis.

In this study the H295R cells were exposed in 48h to three different concentrations, 150 µM, 175 µM and 200 µM, of PFOS and PFNA in 75cm2 tissue culture flasks. The viability was measured with the alamarBlue ® assay, while four hormones related to the steroidogenesis, oestradiol, testosterone, cortisol and progesterone, were measured by using radioimmunoassay. RNA, DNA and proteins were isolated from the exposed cells, and toxic effects on the cell-model were investigated by using proteomics. 2D gels were performed to separate the proteins, and statistically significant proteins spots were excised and identified by LC-MS/MS followed by on-line database searches, using SwissProt and NCBInr databases. Western blot was used to quantitatively validate the findings. In addition, other proteins were chosen for western blot in the light of knowledge of proteins involved in steroidogenesis and cellular stress.

Exposure to PFOS had a cytotoxic effect on the cells at all three concentrations. In addition, exposure to PFOS caused an increased oestradiol at the lowest concentration, but no significant alterations at the two highest concentrations. Testosterone secretion was decreased at the highest concentration, and cortisol and progesterone secretion was decreased at all concentrations. Exposure to PFNA caused a statistically non-significant increased secretion of oestradiol and a statistically significant decreased secretion of testosterone, cortisol and progesterone at all concentrations. Seventeen of the protein spots that showed significant differences in normalized volumes were identified and included proteins involved in transcription/protein synthesis, transport, and stress response. The western blots showed increased vimentin in cells exposed to PFNA, and decreased levels of BAX were found in cells exposed to PFOS.

In conclusion, exposure to PFOS and PFNA were found to alter adrenal steroidogenesis and protein expression in the H295R *in vitro* model. Although the mechanisms of these alterations are not known, the current study might contribute in a better understanding of effects of PFOS and PFNA. The alterations in protein regulation suggested that exposure to these compounds affects several cellular processes, including protein synthesis, stress response and apoptosis. In addition, many of the altered proteins have been reported to be involved in carcinogenesis.

Contents

Abbreviations

µg	Microgram
µl	Microliter
µM	Micro molar
1D	One dimensional
2D	2 dimensional
3β-HSD	3-β-hydroxysteroid dehydrogenase/Δ-5-4 isomerase
ACTH	Adrenocorticotropic hormone
ACTL6A	Actin-like 6A isoform 1
ADP	Adenosine diphosphate
AII	Angiotensin II
ALB	Albumin
ATP	Adenosine-5'-triphosphate
ATP5A1	ATP synthase, alpha 1
BAF	Bioaccumulation factor
BAX	Bcl-2–associated X protein
BCF	Bioconcentration factor
BMF	Biomagnification factor
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CDSE1	Cold shock domain-containing protein E1
C-F bonds	Carbon-fluorine bonds
Cort	Cortisol
CRT	Corticotropin-releasing hormone
CYP11A	20,22-desmolase
CYP11B2	Aldosterone synthase (or 18-hydroxylase)
CYP17	17α-hydroxylase
CYP19	Aromatase
CYP21	Hydroxylase
DDT	Dichloro-diphenyl-trichloroethane
dH2O	Destilled water
DHEA	Dehydroepiandrosterone
DMSO	Dimethyl sulfoxide

DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
E2	Oestradiol
EDC	Endocrine disrupting chemicals
eEF2	Eukaryotic translation elongation factor 2
eIF3M	Eukaryotic translation initiation factor 3, subunit M
eIF4A	Eukaryotic initiation factor-4A
eIF4A1	Eukaryotic initiation factor-4A1
eIF4A2	Eukaryotic initiation factor-4A2
ER	Endoplasmatic reticulum
eRF1	Eukaryotic class 1 release factor
EWSR	Ewing sarcoma breakpoint region 1 protein
FMI	High Mobility Group protein B1
FTOH	Fluorotelomer alcohol
GJIC	Gap junction intercellular communication
H295R	Adrenocortical carcinoma cell-line
HMG	High Mobility Group
HMGR	3-hydroxy-3-methylglutaryl-CoA-reductase
HMGRB1	High Mobility Group protein B1
HPA-axis	Hypthalamus pituitary adrenal axis
HSP70	Heat shock protein 70 kDa
HSPA5	Heat shock protein 70 kDa protein 5
i. d.	Inner diameter
IPA	Ingenuity Pathway Analysis
kDa	Kilo Dalton
KLIF	Norwegian Pollution Control Authority
Kow	Water octanol coefficient
LC-MS/MS	Liquid chromatography - mass spectrometry/mass spectrometry
LDL	Low density lipoproteins
NEDD8	Neural precursor cell expressed, developmentally down-regulated 8
NF-KB	Nuclear factor kappa-light-chain-enhancer
nM	Nano molar
nm	Nanometer

NOAEL	No observed adverse effect level
P4	Progesterone
PABPC1	Poly(A) binding protein 1
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with tween
PCB	Polychlorobiphenyls
Pdcd4	Programmed cell death protein 4
PDIA4	Protein disulfide isomerase A4 precursor
PFC	Perfluorinated compounds
PFDA	Perfluorodecanoic acid
PFDoA	Perfluorododecanoic acid
PFNA	Perfluorononanoic acid
PFOA	Perfluoroanoic acid
PFOS	Perfluorooctanesulphonate
PFTE	Polytetrafluoroethylene
pg	Pikogram
PI	Isoelectric pH
PPAR	Peroxisome proliferator-activated receptor
PRTFDC1	Phophoribosyl transferase domain containing 1
RIA	Radio Immuno Assay
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulfate
SREBP	Sterol response element binding protein
StAR	Steroidogenic acute regulatory protein
T	Testosterone
TBA	Tetrabutyl ammonium salt
TCDD	Tetrachlorobenzo-p-dioxin
TPI1	Triose-phosphate isomerase
UBA3	NEDD8-activating enzyme E1
VIM	Vimentin

1. Introduction

1.1 Worldwide exposure to persistent organic pollutants (POPs)

Persistent organic pollutants (POPs) are a group of chemicals which have recently become a great concern in the environment. They are carbon-based and are primarily products and by-products of industrial processes. Among the most well-studied compounds are polychlorobiphenyls (PCBs), dioxins such as tetrachlorobenzo-p-dioxin (TCDD) and organochlorine pesticides such as dichloro-diphenyl-trichloroethane (DDT) (Corsolini et al. 2005). Most of the POPs resist photolytic, biological and chemical degradation, and some of the substances are semivolatile (Fisher 1999). The POPs have been used for several years, and occurrence of these substances in humans and in the environment have been reported since the early 1960s (Li et al. 2006). Due to their resistance of degradation, the POPs bioaccumulate in the environment, and are able to be transported far distances from the point-sources. The POPs are often made volatile in tropic areas with relatively high temperature and are transported and deposited worldwide (Li et al. 2006). This way of transportation is called the "grasshopper effect", and the process is often seasonal. The substances are released into the environment as for example vapor, and are deposited another place in the world through for example a rainfall (Fisher 1999). POPs have been found in organisms in the Arctic regions where there are few sources of contaminations, as well as in heavily populated, industrial areas.

A common problem occurring when the POPs are tried to be replaced in industrial purposes, is the developing countries' disability to take the cost for the replacement. The alternative chemicals for POPs are often more expensive, and there has not been done enough research for the health effects for some of the replacing chemicals (Fisher 1999).

Some of the toxic effects that have been associated to exposure to POPs are cancer, reproductive and developmental problems such as low birth weight, hormone alteration, lower IQ and emotional problems, alterations of the immune system, endocrine disruption, central nervous system defects, effects on the nervous system, liver damage, skin and eye disease, and death (Corsolini et al. 2005).

Monitoring of humans has shown that POPs continue to be present in most food chains worldwide. Even if concentrations in the environment of some POPs have been reduced since the 1960s and 1970s, both human and animal exposure continues. The reductions in

discharge of toxic substances are not necessarily reflected in measured concentrations in animal and human populations due to many POPs ability to increase with the age of the exposed organism. So even if the exposure decreases, the average concentration in a population will stay the same (Porta et al. 2008).

Most POPs are stored in fatty tissue, due to their lipophilic properties. Nearly all humans have POPs in various concentrations in their livers and other fatty tissues. Although many of the POPs have been restricted or banned, levels of these POPs can still be found in the environment. The most common source of contaminants for human exposure is via the diet, specially via fat fish and fatty tissues from animals (Porta et al. 2008).

The potential of a substance to bioaccumulate in an organism is expressed either as the bioaccumulation factor (BAF), the bioconcentration factor (BCF), or by using the octanol-water coefficient (K_{OW}). BAF and BCF are calculated by measuring the total concentration in an organism and compare this to the surrounding medium. The K_{OW} is a value for how much of a substance that solves in water compared to octanol. If a substance is more soluble in octanol, this means that it is more lipophilic and have the potential to accumulate in lipids (Li et al. 2006).

1.2 Perfluorinated compounds (PFCs) – History and utilization

Perfluorinated compounds (PFCs) are man-made chemicals, and are rarely found naturally. They have been used since the 1950s due to their unique physic-chemical properties such as chemical and thermal stability, low surface free energy and surface active properties (Fromme et al. 2009). In addition the PFCs are both fat and water repelling, due to their structure, and are typically used as surfactants in non-stick products, stain-resistant fabrics and all-weather clothing (Skoog et al. 2008). The PFCs have also been used in food packaging and flame retardant foams (Bjork et al. 2008) as well as in electronic and photographic devices, cosmetics, and surfactants in various cleaning agents (Fromme et al. 2009).

PFOS and PFOA are the two most well-known and most studied PFCs found both in the environment and in humans. PFOS is found to be most abundant (Jensen et al. 2008).

The Organisation for Economic Co-operation and Development (OECD) had several meetings from 2000 to discuss the rising concern about the hazards of PFOS and its salts. The Chemical Committee and the Working Party on Chemicals, Pesticides and Biotechnology made an assessment, and this document was published in 2002 (ENV/JM/RD(2002)17/FINAL 2002). In the same time period, the major manufacturer, 3M, decided to phase out the production of PFOS and PFOS-related substances due to the findings of PFOS dispersed in the environment, including in humans (3M 2000). The phase out of production of PFOS was decided in May 2000 and was completed in 2002.

In 2001 an international treaty was signed to control the release of POPs into the environment. This treaty is called the Stockholm Convention on Persistent Organic Pollutans. This treaty originally listed twelve chemicals of concern; “the Dirty Dozen”. These chemicals are monitored internationally to be eliminated, to restrict the use and/or to reduce release into the environment. The Stockholm Convention decided in Geneva in May 2009, to include PFOS and its salts in addition to “the Dirty Dozen” (Wang et al. 2009).

The production of PFOS and PFOS-related product has decreased, but due to specialized industrial processes with no possible replacements, PFOS is still being produced in some countries. The regulations are very different in countries worldwide when it comes to releasing POPs into the environment, so countries with less stricter regulations have taken over the production from industrial countries with stricter regulations including the U.S. (Wang et al. 2009).

In 2005 the environmental authority in Norway worked out a plan of action for perfluorinated compounds. This included initiatives to reduce the discharge of PFOS and plans to increase the health- and environmental knowledge when it comes to the properties of other perfluorinated compounds. A prohibition against the use of PFOS and PFOS-related compounds in firefoam, textiles and impregnating agents were introduced in Norway in April 2007. In 2008 The Norwegian Pollution Control Authority (KLIF) published a revised plan of action for the work with reducing the perfluorinated compounds (KLIF 2008). This plan has PFOS and perfluoroanoic acid (PFOA) as main focuses since these two compounds are mostly widespread. However, the plan aims at stopping or reducing the discharge of all PFOS- and PFOA like substances.

There is no production of perfluorinated compounds in Norway, and all the products containing these compounds are imported either as chemical-technical products or as component parts in processed products. The most known brands in Norway containing perfluorinated compounds are Gore Tex® and Teflon®. Both of these brands is manufactured from polytetrafluoroethylene (PFTE) and utilizes PFOA (Jensen et al. 2008).

Degradation of fluorotelomer alcohol (FTOH) is likely to be an important source of PFC pollution (Ellis et al. 2004). A study done by Tomy et al. (2004) showed levels of PFOS in livers of rainbow trout exposed to N-Ethyl perfluoroctanesulfonamid suggesting a possible biotransformation route. This may explain the detection of PFOS in fish despite the nonvolatile nature of PFOS (Tomy et al. 2004b).

1.3 H295R – the adrenocortical carcinoma cell line

The adrenocortical carcinoma cell line – H295R – was derived from a human adrenal carcinoma and has all the enzymes necessary for producing hormones. The cells maintain the ability of expressing all the genes needed for production of these enzymes. Thus the H295R cells have the capacity of producing all the hormones involved in the steroidogenesis (Gazdar et al. 1990).

The H295R cell model has shown to help understanding modes of action for several substances, which can reduce the need for laboratory animals as well as being a fast and sensitive tool for screening chemicals (Gracia et al. 2006). In 2006 the first article where the cell model was used to look for endocrine disruption after exposure, in this case to contaminated sediment, was published. The researchers concluded that this model can be used as a model for endocrine disruption, but confirmations must be conducted in *in vivo* models – hence the H295R cells is a good tool for pre-screening (Bláha et al. 2006; Gracia et al. 2007). The model has been compared to fish models to see if it gives satisfactory results when compared with effects on whole organisms. The study showed variations, but the H295R cell model was more consistent and reproducible than the fish models (Villeneuve et al. 2007). The growing focus on reduce, replace and refine studies involving laboratory animals has contributed to make the H295R cell model useful on basal functions and regulation on human adrenocortical steroidogenesis (Oskarsson et al.

2006). In conclusion this cell-line has been frequently used, and today it is a standard model for study of human adrenocortical steroidogenesis (Nicol et al. 2009).

In the current study, alterations of the steroidogenesis in the H295R cells were measured by quantifying four of the steroidogenic hormones; oestradiol, testosterone, progesterone and cortisol. These were chosen due to their importance in reproduction and development.

1.4 Proteomics

The human genome project has recently sequenced the whole human genome, and has provided information about most of the individual genes' sequences. However, genomics do not give information about the dynamic cellular processes such as the post-translational modifications of the proteins, which is important for protein function activity, or the amount of active proteins in a cell (Chambers et al. 2000). The genetic material is the basis for production of all cellular proteins. The information includes organization of genes and transcriptional regulatory elements on chromosomes, which contributes to understanding the gene functions and their role in specific processes (Heijne et al. 2005). Genomic analysis measures the mRNA levels which provide information on gene expression, but these levels do not necessarily correlate with protein abundance. The levels of mRNA and the corresponding protein may vary a great deal (Rohrbough et al. 2007). The lack of correlation may be caused by post-translational modifications, as already mentioned, such as phosphorylation, acetylation, glycosylation, ubiquitination etc. which may regulate the proteins. Another limitation of genomics is that one gene not necessarily codes for one protein. The proteins contain polymorphisms that may cause different functions. In addition many mRNAs are known to be differentially spliced, generating several proteins (Barrier et al. 2005).

Proteomics however, gives the opportunity to identify post-transcriptional modifications and protein interactions in addition to identification of proteins. This contribute to give a better understanding of disease processes and mechanisms of action (Stigliano et al. 2008) . Proteomics have been defined as the study of protein properties (expression level, post-translational modifications, interactions etc) on a large scale to be able to investigate the global, integrated view of disease processes, cellular processes and networks at a cellular level (Blackstock et al. 1999).

Proteomic analysis measures all the proteins of a cell at a given time. In comparison to the genome which is the same in all the cells, the proteome is different is each cell type in an organism. The proteome will change over time as the organism grows. In addition, infections, use of medication, diet, exercise etc will alter the proteome as response to fluctuations in the intracellular and extracellular environments (Campbell et al. 2003).

Proteomics is considered more complex than genomics, due to the need of more information to interpret the results. There is need of information about functions of the proteins, their structure, their interactions, and which proteins are present at different life stages. In comparison, the genome is the same in all the cells and alterations caused by for example mutations are easier to discover (Campbell et al. 2003).

A difficulty concerning protein analysis is to detect low abundance proteins compared to the more abundant ones. In addition the smaller proteins can be difficult to detect as larger proteins appear more clearly (Rohrbough et al. 2007).

There are several ways of quantification and identification of proteins. The methods used in this study will be further described in the background chapter.

1.5 Aims of the study

The aims of this study were to investigate effects of the perfluorinated compounds perfluorooctanesulphonate (PFOS) and perflouorononanoic acid (PFNA) on protein expression and endocrine function in the human adrenocortical carcinoma cell line – H295R.

Specific aims:

1. To investigate protein expression in response to PFOS and PFNA exposure in the adrenocortical carcinoma cell line H295R
2. To investigate endocrine responses to PFOS and PFNA in the cell line H295R by measuring alterations in four of the hormones involved in steroidogenesis; oestradiol, testosterone, progesterone and cortisol
3. To validate proteomic results after exposure to PFOS and PFNA in H295R cells using western blot
4. To quantify proteins involved in steroidogenesis and cellular stress using western blot

2. Background

2.1 Perfluorinated compounds (PFCs)

2.1.1 Structure of PFCs

The PFCs are built up of a chain of carbon that makes up the linear backbone of the molecule. The molecule consists of typically 4-14 carbon atoms, and the hydrogen atoms are replaced by fluorine atoms (Lau et al. 2007). Fluorine is one of the most reactive atoms in ionic form, and one of the most stable atoms in bound form (Lau et al. 2004). A functional group is attached to the backbone of carbon, and gives the compound its chemical properties. Due to the functional group, the perfluorinated compounds are both hydrophilic and hydrophobic; the perfluorinated alkyl chain is hydrophobic and the functional group is hydrophilic (Fang et al. 2010). The most abundant functional groups are sulfonate and carboxyl. Thus the two main groups of PFCs are perfluorinated sulfonates, including PFOS, and perfluorinated carboxylic acids, including PFNA (Haukås et al. 2007).

The last years PFCs have emerged as a concern in the environment because of their stability and persistence in the environment (Lau et al. 2007). The stability is mostly caused by the replacement of all or some of the carbon-hydrogen bonds by carbon-fluorine bonds (C-F bonds). The C-F bonds are very strong and are resistant to various degradations such as reactions with acids and bases, oxidation and reductions (Fromme et al. 2009) as well as resistance to hydrolysis, photolysis, microbial degradation and living cell metabolism (Kleszczynski et al. 2007). The fluorine is highly electronegative and will attract the electrons in a chemical bond toward itself. This gives the C-F-bonds polarity and strength. In addition, fluorine has three pairs of electrons not involved in binding to other atoms which gives highly fluorinated systems high thermal and chemical stability (Giesy et al. 2002). Naturally occurring PFCs are rare due to the requirement of high energy to break or form the C-F bonds (Lau et al. 2004). Naturally occurring PFCs usually contain one fluorine atom and are referred to as fluorinated organic compounds (FOCs), while the synthetic PFCs often contain several fluorine atoms and might also be fully fluorinated (Giesy et al. 2002). Due to these C-F bonds, PFCs have been considered to be metabolically inert and non-toxic (Hu et al. 2005).

A study done in rats concluded that PFCs with longer carbon chain length have greater potential to accumulate in the liver. In addition longer perfluoralkyl chains are likely to be more potent to peroxisomal β-oxidation (Kudo et al. 2006). The length of the carbon chain has also proven to affect the elimination rate of perfluorinated fatty acids. Longer carbon chains give slower elimination through urine. Thus PFNA has a slower elimination rate than PFOA (Kudo et al. 2001).

Field-based and laboratory-based studies reported that BMFs and BAFs increase with increasing perfluoralkylated chain (Houde et al. 2006).

PFCs have been reported to have various effects in several organisms due to their structure. Perfluorinated fatty acids have structural similarity to natural endogenous fatty acids. The combination of this and surface acting physio-chemical properties and membrane-related effects, the PFCs have attracted interest in studies of effects of permeability status of the cell membrane (Hu et al. 2003).

Previous studies have suggested that perfluorinated compounds have an effect on gap junction intercellular communication, and some of the effect may be due to alterations of membrane fluidity. The membrane fluidity affects movement of molecules in the membrane, and thereby affects lipid-lipid, lipid-protein and protein-protein interactions. Alterations of the membrane fluidity caused by xenobiotic compounds may have effects on the functions of the membrane and its associated substructures (Hu et al. 2003).

PFC's are peroxisome proliferating compounds, and are found to be agonists to the peroxisome proliferator-activating receptors (PPAR). This may affect anti-inflammatory and immunomodulatory actions such as nuclear factor-kappa B (NF-KB) signaling pathway. This pathway is a DNA binding-independent interference of transcription factors (Fang et al. 2008).

The perfluorinated compounds has shown to give developmental neurotoxic effects *in vivo* in the form of a direct action on replicating and differentiate neurons (Slotkin et al. 2008).

PFCs have been found to have three basic effects of mitochondrial bioenergetics; nonspecific increase in ion permeability of the mitochondrial membrane, ionizable amides may cause a specific and potent uncoupling of mitochondrial oxidative phosphorylation, and interference of mitochondrial bioenergetics by inducing

mitochondrial permeability transition, leading to inhabitation of ATP synthesis and release of apoptogenic factors (Starkov et al. 2002).

Certain fluorotelomere alcohols are suggested to induce hepatic vitellogenin mRNA and protein via estrogen receptor signaling in male medaka. In mammals some of the fluorotelomere alcohols are metabolized to PFOA and PFNA, which induce peroxisomal proliferation (Ishibashi et al. 2008).

2.1.2 Perfluorooctane sulfonate (PFOS)

Perfluorooctane sulfonate (PFOS) is a perfluorinated compound which contains an eight-carbon backbone as shown in Fig 2.1. It appears to be the final degradation product of many of the commercially produced PFCs (Krøvel et al. 2008).

PFOS does not decompose in nature, and PFOS related compounds have proved to up-concentrate in the food chain. Studies have shown that PFOS is toxic to repeated exposures, has reproductive effects on mammals and might be carcinogenic (TA-2395/2008, (KLIF 2008).

PFOS is primarily absorbed and distributed in the serum and the liver, but poorly eliminated (Lau et al. 2004). The substance binds to proteins rather than to fatty lipids as opposed to many other POPs (Wang et al. 2009).

PFOS has shown to give diverse effects in laboratory animals including decreased body weight, enlarged liver, hepatotoxicity, decreased serum cholesterol levels, teratogenicity, neurotoxicity, induced peroxisome proliferation and endocrine disruption. However, most of the effects on laboratory animal occur after exposure to higher concentrations than found in the environment (Wang et al. 2009).

Exposure of PFOS has demonstrated both maternal and developmental toxicity in rat and mouse. The maternal toxicity includes deficits in weight gain during pregnancy and increased liver weight (Thibodeaux et al. 2003). In subchronic toxicity tests of PFOS in cynomolgus monkeys, the effects included decreased body weight, increased liver weight, lowered serum total cholesterol concentrations, lowered triiodothyronine concentrations and lowered estradiol levels (Seacat et al. 2002).

In various species of fish, PFOS has shown capability of inducing oxidative stress. This suggests that some of the toxicity of PFOS might be associated with reactive oxygen species (ROS) (Oakes et al. 2005).

In a study done by Hu et al. (2003), PFOS was reported to increase the membrane permeability of cells to two model compounds, TCDD and E2. The amount of the two ligands reaching the cell signaling pathways were increased while PFOS itself were inactive. In addition PFOS was able to decrease mitochondrial membrane potential in exposed cells (Hu et al. 2003).

There is found high levels of PFOS related compounds in the blood of exposed workers in USA. Also relative low levels of PFOS related compounds are found in the regular population several places in the world (TA-2395/2008, (KLIF 2008).

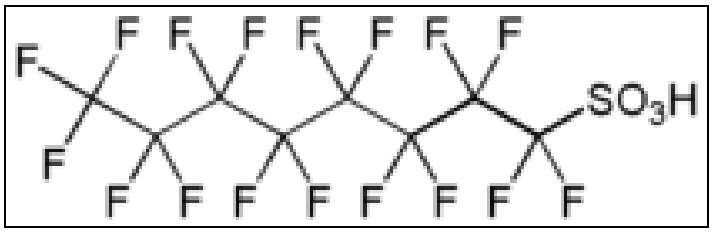


Figure 2.1: An illustration of the structure of perfluorooctane sulfonate.

2.1.3 Perfluorononanoic acid (PFNA)

Perfluorononanoic acid (PFNA) contains a nine-carbon backbone as shown in Fig 2.2, and is the third most abundant PFC in the environment. PFNA can be bioaccumulated and biomagnified through food webs, and in animal experiments it has shown potential reproductive interferences. PFNA has been found throughout wildlife species such as amphipods, fish, birds and mammals including humans. The source of PFNA is not known, but a likely source is atmospheric FTOH emitted through industrial activities which produces perfluorinated carboxylic acids upon degradation (Fang et al. 2008).

PFNA is different from PFOS and PFOA due to the nine perfluorinated carbonatoms in the chain of alkyls, instead of eight. This is likely to make PFNA more toxic than the other two compounds (Fang et al. 2009).

Due to the increasing knowledge of PFOS' toxicity, some fluoropolymer industries use PFNA instead of PFOS and PFOA in the manufacturing of fluoropolymers such as polyvinylidene. A consequence of this is the finding of high concentrations of PFNA in organisms (Fang et al. 2010). Another reason is that the longer-chain PFAAs showed more bioaccumulation than other shorter homologues (Alexander et al. 2007).

PFNA has shown to promote apoptosis in rat spleens. The mechanism behind this may be the activating of PPARs which leads to increased peroxisomal β-oxidation and the production of excess H_2O_2. Decreased activity of superoxide dismutase has also been found, and contributes to the damage caused by oxygen species. In addition increased levels of cytokines have been found in the rat spleen, suggesting an increased apoptosis (Fang et al. 2010).

There are no reports about adverse clinical effects from occupational exposure to PFNA (Mundt et al. 2007).

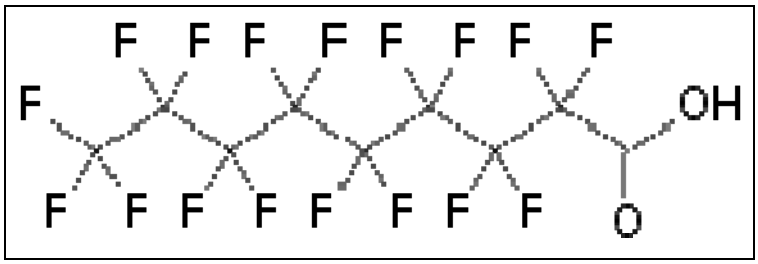


Figure 2.2: Illustration of the structure of perfluorononanoic acid (PFNA)

2.1.4 Exposure in the environment and wildlife

Although PFCs have been used since the 1950s, it has been difficult to investigate the effects in the environment due to lack of measurement techniques. Due to the PFCs surface-active properties, the compounds are more difficult to separate from tissues than other POPs. In addition the compounds demand specialized liquid chromatography/mass spectrometry methods for analysis (Giesy et al. 2001).

PFOS act differently from other POPs when it is released into the environment, including intrinsic properties such as surface activity, water solubility, non-measurable K_{OW} values, and relatively low bioaccumulation potential (Wang et al. 2009).

PFC's have been found in several matrixes, for example surface waters, air, sludge, soils, sediments and ice caps. However water is expected to be the most common compartment where PFC's are found due to their physical-chemical properties. PFC's found in other matrixes are more likely to be associated to the water phase of the compartment and there may be weak electrostatic interactions (Lau et al. 2007).

The dispersion of PFC's is not certain, but there are two theories; long range transport by oceanic currents or atmospheric transport and transformation of precursor chemicals. Anyhow, PFC's are found in less populated areas as well as urban areas (Lau et al. 2007).

In 2001, Giesy and Kannan reported for the first time about the dispersion of PFOS. This report was part of a bigger study were the distribution and effects of PFOS were investigated in tissues from various species, including fish, birds and marine mammals. The tissues were collected from many parts of the world, including urbanized areas in North America, the Arctic and the Baltic Sea, amongst others. The results confirmed that PFOS is widely distributed at a global level, can be persistent and bioaccumulate in food chains (Giesy et al. 2001). The same year PFOS was detected in blood and livers from marine mammals, including dolphin, ringed seal and cetaceans collected from various areas in the world. Interestingly the study showed that PFOS did not seem to increase with the age of the marine animals (Kannan et al. 2001). Another study confirmed this by looking at levels of PFOS in livers from birds. The result was no correlation between PFOS concentrations and sex or age of the birds. One reason suggested was that due to PFOS' affinity for binding to proteins in plasma and liver, the accumulation is controlled by a dynamic equilibrium between uptake and elimination or related protein turnover. In comparison, other POPs accumulate in fatty tissue and are transmitted throughout the food chains (Kannan et al. 2002a). Concentrations of PFOS and related compounds measured in mink in the United States also confirmed no correlation between concentration of PFOS and sex or age. Locations and concentrations of PFOS were compared, and mink living in more urbanized areas with or without industry had greater concentrations of PFOS and its related compounds (Kannan et al. 2002b).

Although there are variations, mammals feeding at higher trophic levels in the food web have higher concentrations of PFCs than the mammals feeding at lower trophic levels. This has been stated in several studies (Giesy et al. 2001; Martin et al. 2004; Tomy et al. 2004a). In marine environment of Greenland and the Faroe Island PFCs, particularly

PFOS, were found to be important contaminants. Concentrations of PFOS were found to follow biomagnification along the food chain, with highest concentrations found in livers from polar bears (Bossi et al. 2005). Occurrence of PFOS in the Great Lakes in the United States was found in all trophic levels of the food chain, with a tendency of biomagnification at higher trophic levels. Due to the better ability of organisms at higher trophic levels of metabolizing the precursor compounds of PFOS, levels of un-metabolized precursors of PFOS in organisms at lower trophic levels serve as a contributory source of PFOS for the organisms at higher trophic levels of the food chain (Kannan et al. 2005). There was not found any biomagnification in aquatic organisms of the food web investigated in the Canadian Arctic (Kelly et al. 2009).

The effect on sediment-dwelling organisms have not been marked, but the organisms might be affected by chronic exposure (Stomperudhaugen et al. 2009).

PFOS has been found to be acute toxic for fish both in saltwater and freshwater in short-term exposure studies. This apply to water-living invertebrates as well (Yamashita et al. 2005).

2.1.5 Exposure in humans

Occurrence of PFCs have been investigated in human tissues since the late 1960s, but it is not until lately when specific analysis of each compound have been developed, the serum of employers in fluorochemical manufacturing industry have been analyzed for PFCs (Kannan et al. 2004).

Since PFCs have most affinity for binding to proteins, the compounds mainly accumulate in organs such as liver, kidney and spleen, but also in testicles and brain. The half-life is different for the various compounds, but are relatively very long for all of them (Jensen et al. 2008). PFCs have a high affinity for binding to albumin in the plasma, and this affinity may be caused by their structure. Interactions between proteins and surfactants are stronger when the compound contains fluorine atoms, which makes the compound more hydrophobic. In addition compounds with longer hydrophobic chains have stronger ability to bind to proteins such as albumin (Li et al. 2009). In addition, the ratios of PFOS between whole blood, serum and plasma have been measured. This showed that the ratio between plasma and serum is 1:1, while the ratio between serum/plasma and whole blood

is 2:1 independent of the levels measured for PFOS. This indicates that serum and plasma is the preferable matrix for PFOS (Ehresman et al. 2007). Elimination of PFCs by the kidneys are poor due to the plasma protein bindings (Harada et al. 2005).

The main source of exposure in humans is assumed to be dietary intake (Fromme et al. 2009). Still, an association between serum levels of PFCs and measured concentrations in the food has not yet been proved. This indicate that dietary intake is not the only source of exposure in humans (Kärrman et al. 2009). Ingestion or inhalation of dust released from treated or contaminated products containing PFOS might be an additional major intake source (Fisher 1999). In addition migration from food-packaging, such as microwave popcorn bags, through metabolism of fluorotelomer-based precursors can be an indirect source for humans (Houde et al. 2006). However migration from cookware has found to be relatively low (Begley et al. 2005).

A study done by Kannan et al. (Kannan et al. 2004) investigated human blood samples from different countries, both industrial and developmental, to see the distribution of the PFCs around the world. The study showed that there were variations between the countries, but PFOS in various amounts was found in serum from all the countries investigated. The same study showed a lack of both gender- and age-dependent accumulation patterns. This may be related to the perfluorinated compounds affinity for lipoproteins rather than neutral lipids. However serum samples from fluorochemical production employees show no substantial changes in serum hepatic enzymes, cholesterol or lipoproteins associated with PFOS concentrations (Olsen et al. 1999).

A study done on blood-samples from Nunavik Inuit adults in Canada showed that consumption of fish and marine mammals, male gender and age were the strongest predictors of PFOS plasma concentrations. The age-dependency suggests that PFOS has bioaccumulative properties like other POPs including PCBs and other organochlorines (Dallaire et al. 2009). The gender differences have been found in several studies, but the faster elimination by females is not consistent. The half-life of PFOA is shorter in male hamsters and cynomolgus monkeys, while it is faster in female rats and there is no difference in mice or rabbits (Lau et al. 2007). It appears that humans have a long half-life of serum elimination of PFOS and PFOA (Olsen et al. 2007).

There have not been many studies investigating the extent of infant exposure of PFCs through breast milk. One pilot study found relative low exposure through breast milk, and

the concentration of PFCs were found to be much lower in the breast milk than in serum. This might be due to the PFCs affinity for proteins which inhibit the transfer to the human milk. This study suggested that further studies must be done to get more certain results (Ehrenstein et al. 2009). Another study indicated the same. PFCs were measured in human liver and milk, where several PFCs including PFOS, PFOA and PFNA were detected in the liver samples and in the serum, while the levels were lower in the milk samples compared to the liver samples (Kärrman et al. 2010).

Several studies indicate that PFOS may be able to cross the placenta barrier, and thus enter the fetus' circulation after maternal exposure. A study done by Inoue et al. indicates that there might be a barrier effect and that PFOS does not completely pass into the fetal circulation (Inoue et al. 2004).

Apelberg et al. (2007) found small negative associations between PFOS concentrations and birth weight and size in humans, measured in cord serum taken from cord blood (Apelberg et al. 2007).

Neonatal exposure of PFOS in mice has shown to affect proteins important for normal brain development. If the exposure happens during a critical step of the brain growth, alterations in these proteins might be one of the mechanisms behind behavioral defects (Johansson et al. 2009).

In serum samples from the Norwegian population, the time trend shows an increased level of eight PFCs, including PFOS and PFOA, from the 1970s followed by a decline in PFOS and PFOA from the 2000s. This suggests that the use of PFCs has been reduced after various reports published around this time period (Haug et al. 2009).

2.2 The human adrenal gland

The adrenal gland is an endocrine organ important in both synthesis of steroid hormones and as part of the sympathetic nervous system. The gland is composed of two distinct endocrine components; the cortex and the medulla. The adrenal medulla synthesizes and secretes the vasoactive amines, adrenalin and noradrenalin (Stevens et al. 2000).

The adrenal cortex is divided in three distinct zones; zona glomerulosa, zona faciculata and zona reticularis. These zones have functionally distinct roles in steroid hormone

production; zona glomerulosa synthesizes mineralcorticoids, zona fasciculata produces glucocorticoids and zona reticularis produces C19 steroids, including testosterone and 17 β-Estradiol. The enzymes in the adrenal cortex involved in steroidogenesis include five forms of cytochrome P450 (CYP11A, CYP11B2, CYP17, CYP19 and CYP21) and 3β-hydroxysteroid dehydrogenase (3β-HSD). These enzymes are distributed in the endoplasmatic reticulum (ER) and the mitochondria (Rainey et al. 2004). All the three zones use pregnenolone as substrate to synthesize their secretory products (Rainey et al. 1994). Pregnenolone is converted from cholesterol by CYP11A in the mitochondria. Cholesterol can be synthesized de novo by steroidogenic tissue, but most of the derived cholesterol in the adrenal gland comes from receptor-mediated endocytosis of plasma low density lipoproteins (LDL). LDL cholesterol esters can either be stored within the cells as liquid droplets, or be converted directly to free cholesterol to be used in production of steroid hormones (Miller 1988). The cholesterol is required for the cell-membranes fluidity and motions, both the plasma membranes and the intracellular membranes. The intracellular cholesterol is mainly regulated by a group of transcription factors that regulate genes involved in the synthesis of cholesterol and fatty acids, called sterol response element binding proteins (SREBP) (Miller 2007).

2.2.1 Steroidogenesis

The full steroidogenesis is illustrated in the Fig 2.3. The first reaction where cholesterol is transformed to pregnenolone is the rate limiting step due to the transport of cholesterol from the outer to the inner membrane of the mitochondria. This transport is regulated by the steroidogenic acute regulatory protein (StAR) which is directly correlated with the steroidogenic activity in the adrenal and gonadal cells (Sugawara et al. 1995). StARs transportation of cholesterol is regulated by 3-hydroxy-3-methylglutaryl-CoA-reductase (HMGR), which is involved in the control of cholesterol biosynthesis. Progesterone is synthesized from pregnenolone by 3β-HSD. Progesterone can be further converted into 11-deoxy-corticosterone by CYP21, which is converted to corticosterone by CYP11B and further converted to aldosterone by the same enzyme. Pregnenolone can be synthesized by CYP17 to 17α-OH-pregnenolone and may be further synthesized to 17α-OH-progesterone by 3β-HSD. 17α-OH-progesterone can also be synthesized by the conversion of progesterone by CYP17, and is synthesized to 11-deoxycortisol by CYP21

and then to cortisol by CYP11B. 17α-OH-pregnenolone may be synthesized to DHEA by CYP17 which is converted to androstenedione by 3β-HSD. Androstenedione may be synthesized by conversion of 17α-OH-progesterone catalyzed by CYP17. Androstenedione can either be converted to testosterone by 17β-HSD or to estrone by CYP19. Both testosterone and estrone can be converted into 17β-estradiol by CYP19 and 17β-HSD respectively.

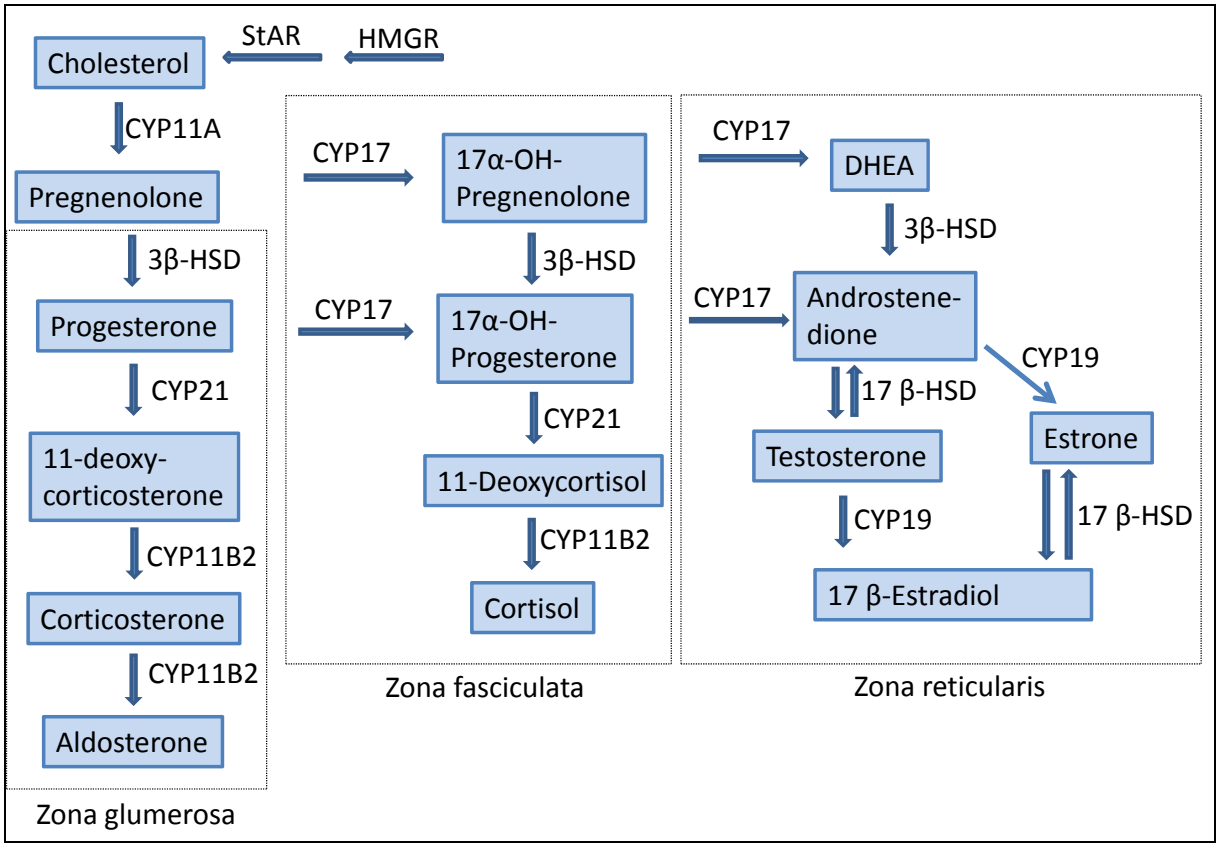


Figure 2.3: Overview over human adrenal steroidogenesis. The boxes contain the hormones, while the names next to the arrow are the enzymes involved. The figure is adapted from (Gracia et al. 2006).

The production of mineralcorticoids in the zona glomerosa is regulated by angiotensin II (AII). The secretion of the glucocorticoids in zona fasiculata is under the control of ACTH, while the secretion of C19 steroids in zona reticularis is regulated by ACTH and forskolin (Rainey et al. 1994).

The production of steroid hormones changes during development, during pathological stress and after exposure to toxicants (Oskarsson et al. 2006).

2.2.2 The HPA-axis

The hypothalamus pituitary adrenal axis (HPA) is a major part of the neuroendocrine system in the human body. Hormones excreted from this axis control reactions to stress, and regulates many biologic processes, including temperature, digestion, the immune system, mood and emotions, sexuality and energy storage. Hypothalamus secretes the hormone corticotrophin-releasing hormone (CRH) which targets the anterior pituitary to release ACTH. ACTH stimulates the adrenal gland to produce cortisol. Cortisol is needed for both anabolic and catabolic metabolism, inhibition of ACTH secretion – negative feedback-loop, vascular reactivity, immune system and inflammatory responses. In addition, cortisol has effects on central nervous system and has effects on stress (Rosmond et al. 2000).

2.2.3 Endocrine disruption

Endocrine disrupting chemicals (EDC) has been defined as "…an exogenous substance or mixture that alters function(s) of the endocrine system and consequently produces adverse health effects in an intact organism, or its progeny, or (sub)populations" (Goksøyr 2006) and as "… an exogenous agent that interferes with the production, release, transport, metabolism, binding, action or elimination of natural hormones in the body responsible for the maintenance of homeostasis and the regulation of developmental processes (Whitehead et al. 2006)." These definitions are wide and include a great number of substances. The focus have mainly been on the nuclear receptor super-family, including the estrogen receptor and the androgen receptor, either as agonists or antagonists of endogenous hormones (Whitehead et al. 2006). More recently many of the chemicals inducing endocrine disruption have found to be non-receptor mediated. They may alter endocrine functions such as chemical-induced modulation of the enzymes involved in the production, transformation or elimination of steroid hormones. This may further alter the concentrations of hormones in blood and tissues. The non-receptor mediated processes can occur indirectly via alterations of common signal-transduction pathways, or directly via competitive or non-competitive inhibition of the steroid enzyme (Hecker et al. 2008). To summarize, the mechanisms of action of EDC are:

1. Agonistic/antagonistic effects – hormone mimics
2. Disruption of production, transport, metabolism or secretion of natural hormones
3. Disruption of production and/or function of hormone receptors

(Goksøyr 2006)

The conservation of the endocrine system across various species (Kavlock et al. 1996).

There are both natural and synthetic sources of EDCs. The natural occurring sources include human and animal hormones and phyto- and mycoestrogens found in for example food, sewage and animal husbandry runoff. The synthetic sources include plastics, detergents, drugs, cosmetics, flame retardants, herbicides and pesticides, and industrial productions (Goksøyr 2006).

2.3 H295R

The human adrenocortical carcinoma cell line NCI-H295 was established from a 48-year-old black woman from the Bahamas with a rare malignant adrenocortical carcinoma. Parts of the tumor were placed in culture in microwells with different types of growth medium. The researchers spent several years optimizing the cell culture, and ended up with cells with the ability to express several multiple pathways of steroidogenesis (Gazdar et al. 1990). The NCI-H295 cell line has shown to act pluripotent and to be capable to produce hormones specific for all the three zones in the adrenal gland (Rainey et al. 1994). It has been proved that the steroidogenetic pathways in the cell lines are based on expression of the same enzymes found in the normal adrenal gland (Staels et al. 1993a). Studies have shown that forskolin and dibutyryl cAMP enhance the production of cortisol, while ACTH was less effective. This indicates that the cells have a decreased expression of ACTH receptors (Rainey et al. 1993). The NCI-H295 cells ability to perform full steroidogenesis in serum-free and cholesterol-free medium, proves that endogenous formation of cholesterol occurs (Gazdar et al. 1990).

The NCI-H295 cell line gave rise to another population of cells; the H295R cell line (Fig 2.4). The difference between these cell lines is the H295Rs capability to form a tightly adherent monolayer in addition to a population doubling time reduced from five to two days (Rainey et al. 2004).

Due to the documentation of the adrenal gland being the most common toxicological target of all endocrine organs (Harvey et al. 2007), the H295R model is a useful tool in the assessment of EDCs. The model has several possibilities including measurement of hormone production (Hecker et al. 2006), gene expression (Hilscherova et al. 2004) and enzymatic activities of steroidogenic genes (Staels et al. 1993b). In addition, a previous study has described the first proteomic study on the H295R cell line (Stigliano et al. 2008).

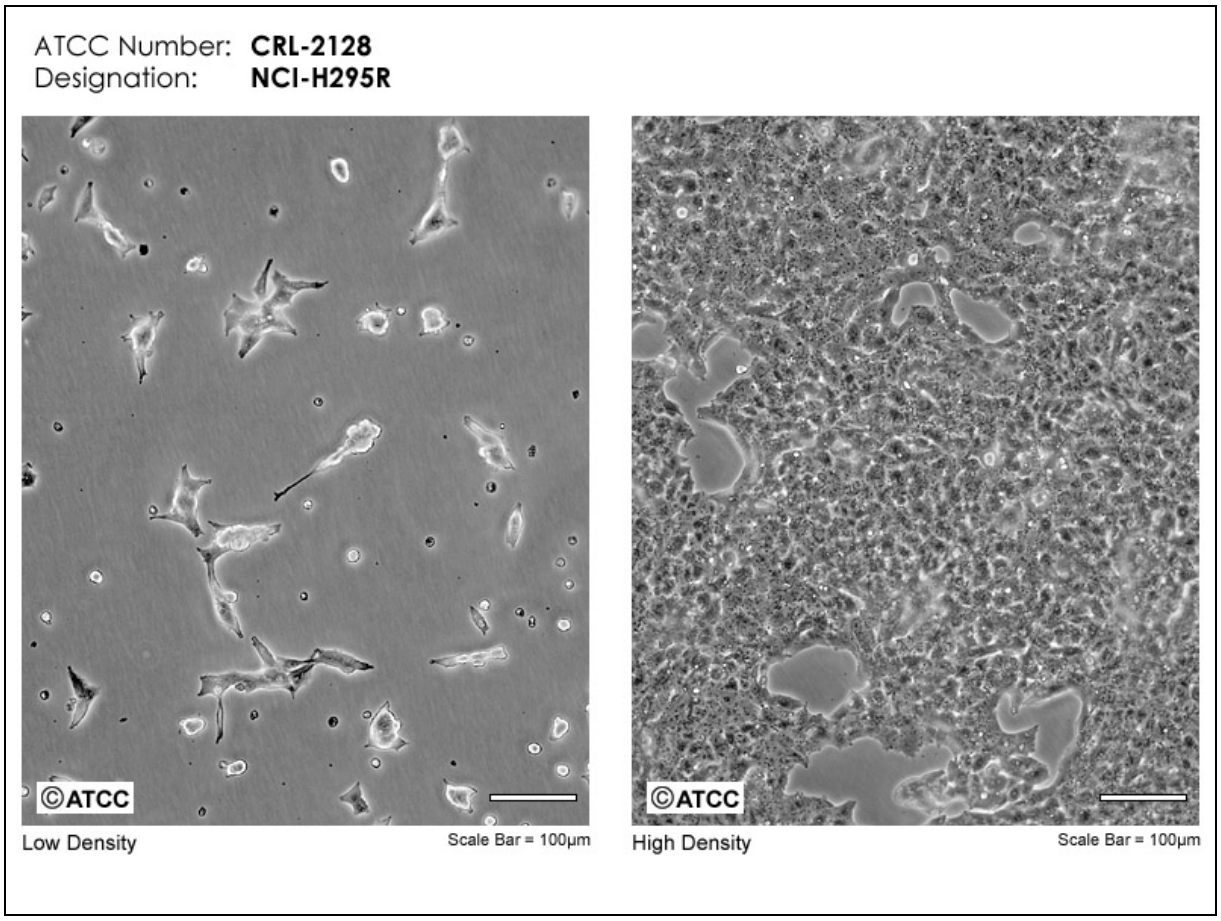


Figure 2.4: Pictures of the H295R cell line obtained from the American Type Culture Collection (ATCC) website (www.lgcstandards-atcc.org). The picture on the left shows the cells growing at a low density, while the picture at the right shows the cells grown in a high density.

2.4 Cell viability

There are several assays to determine the viability of cells. In this study the alamarBlue® assay was used. This assay contains resazurin which is a nontoxic, cell permeable compound that has a blue color without fluorescent abilities. Viable cells convert this

compound to the fluorescent molecule resorufin (Fig 2.5). The amount of fluorescence produced is proportional to the number of living cells (www.invitrogen.com).

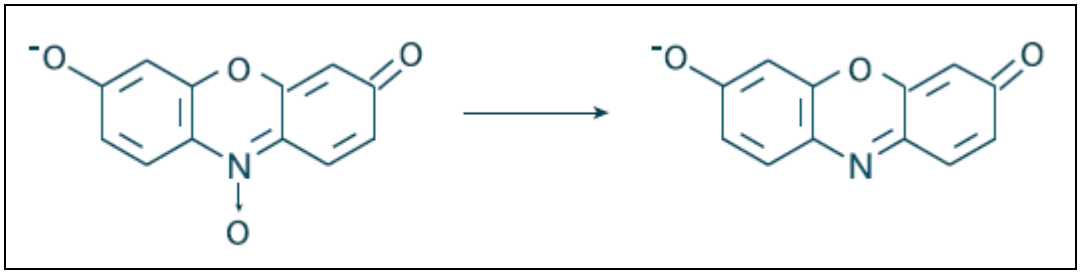


Figure 2.5: The viability assay Alamar Blue® contains resazurin (to the left) which is converted to the fluorescent molecule resorufin (to the right) in viable cells. The amount of fluorescence is proportional to the number of viable cells.

2.5 Protein chemistry

2.5.1 Structure of proteins

Proteins are made up by amino acids. There are in total 20 amino acids which are linked together by peptide bonds in many different ways, making up all the different existing proteins. The proteins have a three-dimensional shape, with four organization levels. The primary organizations is the sequence of amino acids bound together covalently with peptide bonds. These bonds are not broken under conditions that denature proteins, such as heating or high concentrations of urea. To break these bonds, the proteins have to be exposed over time to a strong acid or a strong base at a high temperature. The second organization level is the hydrogen bonds formed between the chains of amino acids, forming α-helixes, β-sheets or β-bends. The tertiary organization level is folding of domains and final arrangements of domains using disulfide bonds (-SH-bonds). While the final organization, the quaternary, is the organization of polypeptide subunits (Champe et al. 1994).

Denaturation of proteins unfolds and disorganizes the protein's structure without breaking the peptide bonds. This is usually an irreversible reaction, but in some rare cases the proteins can reform their originally state. Heat, organic solutions, strong acids or bases, detergents and ions of heavy metals can denature proteins (Champe et al. 1994).

The four levels of organization of the chains of amino acids are the basis of forming specialized functions in the final proteins; the arrangement of the fundamental structural elements in different combinations gives many different groups of proteins such as hemeproteins, fibrous structural proteins, transcription factors and enzymes among others (Champe et al. 1994).

2.5.2 Protein synthesis

The proteins are synthesized in the cells under management of the genome; the DNA. The majority of proteins are synthesized by ribosomes in the cytosol (explained below) or by ribosomes in mitochondria or chloroplasts. When a particular protein is needed in the cell, the specific DNA sequence in a chromosome that corresponds to the needed protein is copied into RNA. These short RNA segments are used as templates to synthesize the protein. The first step in transforming DNA into RNA is called transcription, and begins with opening and unwinding a small portion of the DNA double helix. One of the exposed strands of DNA is used as a template for the synthesis of RNA. The transcription of RNA from DNA is performed by enzymes called RNA polymerase, which catalyzes the formation of the phosphodiester bonds that link the nucleotides, adenine (A), cytosine (C), guanine (G) and thymine (T), together and thus form the sugar-phosphate backbone of the RNA chain. The bases are added one by one by using the DNA as template. Energy for this process is provided by ATP, CTP, UTP and GTP. The RNA segments used in protein synthesis are called messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA) (Alberts et al. 2004).

The conversion of RNA to proteins are called translation due to the information in RNA which is transformed to another language when transferred into proteins (Alberts et al. 2004).

The sequences of nucleotides in the mRNA are read in groups of three, which are known as codons. Since RNA is build up by four nucleotides, there are 4 x 4 x 4 = 64 possible combinations of the three nucleotides in each codon; AAA, AUG, ATG etc. Each codon represents a specific amino acid (Alberts et al. 2004).

tRNA are adaptor molecules that can bind both to the codons and to amino acids. The enzyme aminoacyl-tRNA synthetases make sure of recognition and attachment of the

correct amino acid. This reaction is dependent on energy from hydrolysis of ATP (Alberts et al. 2004).

The main seat in the protein synthesis is the ribosome. This is a large complex composed by many different proteins and several RNA molecules; rRNA. The composition is two-thirds RNA and one-third protein. The ribosome is build up by two subunits, one smaller (30S), and one bigger (50S). mRNA is associated with the 30S subunit which matches the tRNA to the codons of the mRNA. The 50S subunit catalyzes the formation of the peptide bonds that link the amino acids together into a polypeptide chain (Alberts et al. 2004).

The proteins synthesis occurs in three steps; initiation, elongation and termination.

- Initiation: Ribosome binds to the mRNA-initiation complex; mRNA binds to 30S subunit and is joined by the 50S subunit. A peptide bond is formed between the first two amino acids. The initiation always starts with the amino acid methionine, represented by the codon AUG.
- Elongation: All reactions from the first peptide bond to the addition of the last amino acid.
- Termination: The completed polypeptide chain is released, and the ribosome dissociates from the mRNA.

(Lewin 2008)

The final step in the protein synthesis is the recycling of the ribosome. The ribosome is dissociated into its subunits, the mRNA and tRNA are released and the ribosome can be used in another round of translation (Noble et al. 2008).

As described in Fig 2.6, the tRNA brings amino acids, and the growing protein chain is obtained by the interaction of the tRNA that brought the amino acid and the previous amino acid. The ribosome contains two seats for the tRNA; the A-site and the P-site. The A-site is for binding the incoming aminoacyl-tRNA bringing a new amino acid, while the P-site holds the peptidyl-tRNA which is carrying the nascent polypeptide chain. The peptide bond formation between the polypeptide chain and the new amino acids occurs when the polypeptide carried by the peptidyl-tRNA is transferred to the amino acid carried by the incoming aminoacyl-tRNA. This reaction is catalyzed by the 50S subunit of the ribosome. The result is that the peptidyl-tRNA becomes deacetylated in the P-site,

and the aminoacyl-tRNA becomes the new peptidyl-tRNA in the A-site. The ribosome moves one triplet along the mRNA – hence the new peptidyl tRNA is moved from the A-site to the P-site, while the deacetylated tRNA in the P-site is moved to the E-site. The E-site is occupied by the previous deactylated tRNA, which is released into the cytosol when the ribosome moves along the mRNA and can bring the new amino acid to be attached to the polypeptide chain (Lewin 2008).

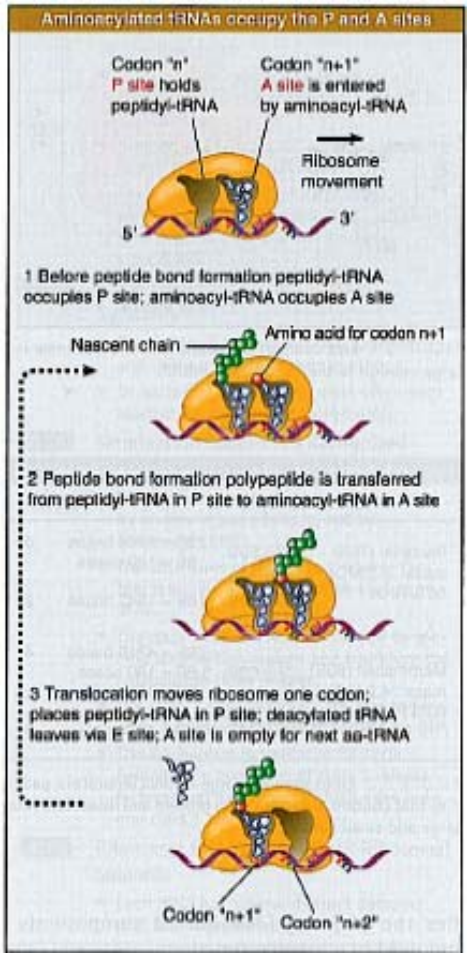


Figure 2.6: A brief overview over the protein synthesis. The synthesis takes place in the ribosomes. The ribosome is build up by proteins and RNA (rRNA). mRNA is bound to the ribosome and gives the code for the specific protein that is to be made. tRNA attaches to the sites in the ribosome special for the tRNA after bringing amino acids to the growing peptide-chain. A start codon induces the synthesis, while a stop codon ends the synthesis. The result is a new protein. This protein undergoes post-synthetic transformation and is transported to the location specific to their functions. Figure is obtained from (Lewin 2008).

The synthesis of the polypeptide chain is terminated by one of the three stop codons UAA, UAG or UGA. These termination codons lie directly after the codon representing the C-terminal amino acid of the polypeptide chain, and are needed for termination of the protein synthesis (Lewin 2008).

There are several factors involved in the various steps of the protein synthesis. Initiation factors are required for all of the steps in initiation, and there are at least 12 of these factors that are directly or indirectly involved. Eukaryotic initiation factor 2 (eIF2) and eIF3 bind to the smallest ribosome subunit, eIF4A, eIF4B and eIF4F bind to the mRNA, and eIF1 and eIF1A bind to the ribosome subunit-mRNA complex. The elongation factor eEF1α mediates the entry of aminoacyl-tRNA to the A-site, while the elongation factor eEF2 helps translocation of the ribosome along the mRNA. The termination codons are recognized by protein release factors; the eukaryotic class 1 release factor, eRF1, recognizes all the three termination codons (Lewin 2008).

The newly synthesized protein are transported within the cell, or out of the cell, associated to their final functions (Lewin 2008).

2.5.3 Groups of proteins

Proteins can be grouped by their functions. Some of these groups are described below.

Enzymes are catalysts that increase the speed of a chemical reaction, and are not consumed during this reaction – thus it can be used in several following reactions. These proteins have an active site formed as a special pocket or a cleft where it is room for the substrate. This active site makes the enzymes highly specific to certain substrates. The catalytic efficiency says something about how long the enzymes use from catalyzing a reaction until the enzyme is ready for a new substrate. Some enzymes need cofactors to be able to perform a chemical reaction. Most enzymes can both be activated or inhibited by various molecules as part of a feed-back system. Enzymes can be divided into six main groups: Oxidoreductases, Transferases, Hydrolases, Lysases, Isomerases and Ligases (Champe et al. 1994).

Structural proteins have the function to provide mechanical support to cells and tissues. Examples of structural proteins are:

- collagen and elastin – constituents of extracellular matrix, form fibers in tendons and ligaments outside the cells
- tubulin – forms long, stiff microtubules

- actin – forms filaments that underlie and support the plasma membrane
- keratin – forms fibers that reinforce the epithelial cells, major protein in hair and horn

(Alberts et al. 2004)

Transport proteins carry small molecules or ions. Serum albumin carries lipids, and is one of the major transport proteins in the blood stream together with hemoglobin that carries oxygen and transferrin that carries iron. Some of the transport proteins are embedded in the membranes to carry small molecules and ions across the membrane (Alberts et al. 2004).

Motor proteins generate movement in cells and tissues. Two example are myosin in skeletal muscle cells that provides the possibility for humans to move, and kinesin that interact with microtubules to make organelles able to move in the cell (Alberts et al. 2004).

Storage proteins function is to store small molecules or ions. For example iron is stored bound to ferretin in the liver (Alberts et al. 2004).

The signal proteins carry signal from cell to cell. This includes many hormones and growth factors that coordinate physiological functions in animals. Insulin is an example, a small protein that controls the glucose levels in the blood (Alberts et al. 2004).

Receptor proteins detect signals and transmit them to the cell's response machinery. An example is the insulin receptor that allows the liver cells to take up glucose in respond to the hormone insulin (Alberts et al. 2004).

The function of the proteins that act as transcription factors is to bind to DNA to switch genes on or off. Homeodomain proteins (HMG) are part of a large group of transcriptions factors that are a part of controlling development in multicellular organism by acting as genetic switches (Alberts et al. 2004).

2.6 Protein analysis

2.6.1 Two dimensional gels (2D-gels)

A two dimensional gel electrophoresis is a combination of isoelectric focusing and sodium dodecylsulfat- (SDS) polyacrylamide gel electrophoresis. The proteins are separated according to their isoelectric point (pI) by isoelectric focusing in the first dimension, and by their molecular weight in the second dimension. The result is a gel containing numerous spots separated from each other (Adams et al. 2005).

The isoelectric focusing is a method to separate the proteins according to their isoelectric point (pI). Due to proteins amphoteric nature – they carry either positive, negative or zero net charge depending on the pH in the surroundings – they have an isolelectric point (pI) at a specific pH where the net charge is zero. Below their pI, the proteins are positively charged, and above their pI, the proteins are negatively charged. In isoelectric focusing, the pH gradient is an important factor because the proteins will migrate to the point where their net charge is zero. Hence, proteins with a positive net charge will migrate towards the anode, and proteins with a negative net charge will migrate towards the cathode (Adams et al. 2005).

The second dimension is usually performed on polyacrylamide gels with denaturing conditions obtained by using an anionic detergent such as SDS. The polyacrylamid gels are generated by polymerization of acrylamide monomers into long chains. These long chains form cross-links between bifunctional compounds such as N, N-methylene bisacrylamide (bis) and free functional groups at chain termini. The pore size of the gel is determined by the concentrations of acrylamide and bisacrylamide – higher acrylamid concentrations gives smaller pore size. The anionic detergents such as SDS denaturate and unfolds the proteins by wrapping around the hydrophobic parts of the protein. The SDS-protein complexes are highly negatively charged, and migrated through the gel based on their size rather than by their charge. If a reducing agent such as dithiothreitol (DDT) is added to the system, the denatured proteins are completely unfolded (Invitrogen 2003).

2.6.2 Liquid chromatography – tandem mass spectrometry (LC-MS/MS)

Liquid chromatography is used to separate substances based on their characteristic polarities and their interaction with the stationary phase. The separation is based by the distribution between a stationary phase and a liquid, mobile phase. The system is build up by several components; a column (the stationary phase), a solvent (the mobile phase), a pump, an injector and a detector. The mobile phase will carry the analytes in the sample past through the column – the stationary phase. As the mobile phase flows through the column, the analytes in the sample will distribute between the two phases. Analytes with more affinity to the column will be held back and travel slowly through the stationary phase, while analytes with lower affinity for the stationary phase will travel faster. This separates the analytes with high affinity for the stationary phase from the analytes with lower affinity for the stationary phase. If an analyte have a very strong affinity for the stationary phase, the physical or chemical nature of the mobile phase must be changed to displace the analyte from the stationary phase to be able to separate analytes in a sample (Burtis et al. 2001).

Mass spectrometry (MS) is a method where the target molecule first is ionized into ions with a specific mass-to-charge (m/z) ratio, and then the ions are measured and separated. The result is a mass spectrum where each ions m/z ratio is plotted against the relative abundance in the sample. This data is used to determine structure and concentrations of inorganic and organic compounds. It has been more difficult to combine MS with liquid chromatography than with gas chromatography because the analytes are solved in a liquid, while the mass spectrometer requires the analyte to be in the gas phase. Four elements have been used to help the possibility to combine liquid chromatography and MS; nebulization of the liquid phase, removal of the bulk solvent, dissociation of the solvent-analyte clusters and ionization of the analytes. This has made the combination of liquid chromatography and MS much easier, and the result is production of ions with minimal fragmentation due to soft ionization (lower energy) (Burtis et al. 2001).

MS requires a transformation of a neutral atom or molecule to an ion created by an ionization step. Many techniques can be used, but for proteins and peptides electrospray ionization (ESI) is a common method. ESI which is most effective for ionic or extremely polar compounds, is able to ionize macromolecules and able to produce multiple charged ions (Burtis et al. 2001). ESI produce charged droplets by passing a solubilized sample

through a high voltage needle at atmospheric pressure. Prior to the entry of the mass spectrometer, desolvation of the samples occurs (Wysocki et al. 2005). The mass analyzer separates the ions according to their m/z ratio before the ions reach the detector. There are several different types of mass analyzers such as magnetic-sector, quadropole mass filter, quadropole ion trap, time-of-flight and ion cyclotron analyzers (Burtis et al. 2001).

An ion trap stores the ions and this capability is used when two or more mass analyzers are operated in tandem. Tandem mass spectrometry (MS/MS) is a technique where mass-specific isolations are done several times in continuous operations. The targeted compound is ionized selectively, and its ions are separated from the other compounds. The selected ions collide with molecules of a neutral gas in a collision chamber resulting production of fragments to be separated and identified in the second spectrometer (Burtis et al. 2001). The first mass-selective ion separated is called the parent ion. The second operation is to determine the mass/charge ratio of the fragment ions produced by collision-induced dissociation of the parent ions (March 1997).

The separation of proteins and peptides by chromatography before reaching the mass spectrometer, helps detecting more ions.

The identification of proteins using mass spectrometry requires interplay between how molecules are ionized, activated and detected, and the gas phase peptide chemistry – which bonds are broken, at what rate, and how cleavage depends on factors such as peptide/protein charge state, size, composition and sequence (Wysocki et al. 2005).

2.6.3 Western Blot

Western blot is a technique that detects proteins in a given sample of proteins extracted from tissue or cells. The proteins are separated by gel electrophoresis by the length of the polypeptides. Secondly the content on the gel is transferred to a membrane, and the proteins are detected by using antibodies specific to target proteins. The targeted proteins are detected by for example a fluorescent secondary antibody. After detection, the proteins can be quantified to see how much is accumulated (Burtis et al. 2001).

3. Material and methods

All the equipments, kits, chemicals, commercial buffers, instruments and softwares are listed in Appendix 1 together with manufacturer. Thus this will not be mentioned in the description of material and methods.

3.1 Cell-line and cultivating cells

The human adenocarcinoma cell-line H295R was obtained from the American Type Culture Collection. The cells were cultivated in 75 cm^2 flasks with 12.5 ml of supplemental medium at 37 °C with a 5 % CO_2 atmosphere. The cells were grown in a 1:1 mixture of Dulbecco's Modified Eagles Medium and Ham's F-12 Nutrient mixture (DMEM/F12) supplemented with 5 ml/l of ITS+ Premix, and 12.5 ml/l of BD Nu-Serum as described earlier (Gracia et al. 2006; Hecker et al. 2006). The medium was refreshed three times a week and sub cultured once a week to maintain cell density and viability. The cells were detached with 2 ml 0.25 % trypsin/0.53 nM EDTA. The detached cells were added to 10 ml medium and centrifuged at 1250 rpm in 5 minutes. The pellet was resolved in medium, filtered through a 70 µM Cell strainer and cultured in 75 cm^2 flasks. Cells between passage 5 and 13 were used in the experiment.

Figure 3.1: Illustration of culturing the H295R cells. The picture to the left shows the sterile cabinet where all the treatment, changing of medium and subculturing of the cells were done. The picture to the right shows the microscope used when checking the cells viability and counting the number of cells.

3.2 Test chemicals

Tetrabutylammonium heptadecafluorooctanesulfonate and perfluorononanoic acid were purchased in powder form from Sigma-Aldrich with a purity of >95% and >97% respectively. Chemicals were dissolved in DMSO to 600nM stock solutions and stored in aliquots at -20°C. DMSO 0.1% was used as a solvent control for PFNA, while tetrabutylammonium cloride (TBA) solved in DMSO 0.1 % were used as a combined solvent and salt control for PFOS. The reason for using a separate control for PFOS was that PFOS were purchased as a tetrabutylammonium-salt. TBA was also compared to DMSO to check whether the salt had an effect by itself.

3.3 Experimental design

The experiment of a PhD student (Kraugerud et al. 2010, manuscript in preparation) was used as template when the experimental design was decided. In her study, 24-well plates were used, and the lowest concentration without toxic effects for PFOS was 600 µM and for PFNA 60 µM. Because of the need of a larger amount of cells to obtain enough proteins for the proteomic analysis, the experiment was conducted in 75 cm^2 flasks. A consequence of changing from 24-well plate to 75 cm^2 flasks was that the ratio between medium and cells hence the ratio between the amount of the compounds and cells, changed. On basis of this, new concentrations were calculated (shown in the table below) to match the amount of compounds per cell used in the previous experiment. The density of cells used in earlier experiments performed in 24-well plates were 150 000 cells per cm^2; each well has a surface of 2cm^2 and holding 1 ml of medium.

Table 3.1: This table shows calculations needed from transformation of a dose-response experiment done in 24-well plate to an experiment in 75 cm^2 flasks, to be able to have similar effects as the previous experiment.

Culture Vessel	Culture Surface (cm2)	cells/cm2	Total Amount of Cells in Vessel	Medium Volume (ml)	Exposure Concentration (µM)	Total Amount (µmole)	µmol/cell
Plate (24-well)	2	150000	300000	1	**60**	0,06	2,00E-07
Plate (24-well)	2	150000	300000	1	**600**	0,6	2,00E-06
Flask (T75)	75	150000	11250000	12,5	**60**	0,75	6,67E-08
Flask (T75)	75	150000	11250000	12,5	**600**	7,5	6,67E-07
Flask (T75)	75	150000	11250000	12,5	**300**	3,75	3,33E-07
Flask (T75)	75	150000	11250000	12,5	**200**	2,5	2,22E-07
Flask (T75)	75	150000	11250000	12,5	**175**	2,1875	1,94E-07
Flask (T75)	75	150000	11250000	12,5	**150**	1,875	1,67E-07

The cells were detached as described above and resuspended in medium. The number of cells was counted by using a Bürker haematocytometer. 4x4 squares were counted, multiplied by two and multiplied by the volume of medium added. After counting, the cells were seeded in the 75 cm^2 flasks at a density of 150.000 cells per cm^2. The cells were exposed to the compounds 24h post seeding. The test compounds, dissolved in DMSO 0.1%, were added to 12.5 ml of medium to give final concentrations of 150µM, 175µM and 200µM for each compound, including the salt control (TBA). Medium containing the compounds was added to the cells. After 48 h, medium was collected and stored at -80 °C for future hormone analysis. The cells were detached as described earlier, and the cell pellets were kept on ice until a lysis buffer (RLT) from Allprep® DNA/RNA/Protein Mini Kit was added to the vials (see further description of isolation below).

3.4 Viability

The viability was controlled visually under a microscope, both 24 h post exposure and 48 h post exposure. The labels on the Tissue Culture flasks were hidden to make the visual observations less biased by not knowing which exposure group that was looked at. To be able to observe any visual effects of the exposures, controls were used in every single experiment.

In addition to visual evaluation, the alamarBlue® Assay was performed on the cells to measure the viability of the cells after exposure. This assay quantifies the proliferation of cells by measuring how much of the reagent is reduced after incubation due to metabolic activity. It is commonly used to check relative cytotoxicity of compounds. After 48 h exposure, the medium was collected and frozen at -80 °C, and replaced with 11.25 ml fresh medium mixed with 1.25 ml alamarBlue®, giving a final concentration of 10 % alamarBlue®. The cells were incubated for three hours under the same conditions as described above. After incubation, the medium was transferred to 96-well plates, and read at 570 nm and 600 nm by a spectrophotometer. The percent viability of the control was calculated according to the manufactures instructions.

The viability assay, alamarBlue®, was done on a separate experiment due to the risk of the reagent in the assay influencing the cells to be isolated. This means that the viability-data is not from the exact same cells that have been isolated and analyzed.

The general tendency showed that the flasks where the cells were exposed to PFOS had a lower density compared to DMSO 0.1 %, TBA and PFNA. In a few of the experiments, all the cells were dead, and these cells have been excluded from further analysis and statistics.

3.5 Isolation of RNA, DNA and proteins

Isolation of RNA, DNA and proteins was done by using Allprep® DNA/RNA/Protein Mini Kit. The manufacturer's protocol was followed, except during resolving the protein-pellet (see description below). In addition, Phosphatase Inhibitor Cocktail 1, Phosphatase Inhibitor Cocktail 2 and Protease Inhibitor Cocktail was added to the buffer RLT (10 µl of each cocktail/1000 µl buffer RLT).

Before starting the protocol, β-mercaptoethanol was added to the RLT buffer (10µl/mL), and 96-100 % ethanol was added to Buffer RPE, Buffer AW1 and Buffer AW2, as described in the manufacturer's protocol. In addition, the Buffer EB was preheated to reach 70 °C.

The cells were trypsinized with 2 ml 0.25 % trypsin/0.53 nM EDTA to detach them from the flasks. Medium was added to inactivate the trypsin/EDTA, and the cells were centrifuged to form a pellet. To lyse the cells, 1 ml buffer RLT, containing the cocktail as described above, was added to the cell pellets from each 75 cm^2 flask. The mixture of buffer and cells were vortexed and added to Qia Shredder columns. Lysate from one 75 cm^2 flask was divided into two Qia Shredder columns due to the high content of cells in each flask. The Qia Shredder columns were centrifugated at full speed for 2 minutes. The flow-throughs were transferred to AllPrep DNA spin-columns, and centrifuged at 10000 rpm for 30 seconds to separate the DNA from the RNA and the proteins. The DNA attaches to the AllPrep DNA spin-columns, and they were placed in new tubes in the fridge for later DNA purification.

The flow-through containing the RNA and the proteins were washed by adding 400 µl of 96-100% ethanol and mixing well without centrifugation. The samples were transferred to

AllPrep RNA spin columns, and centrifuged for 15 seconds at 10000 rpm. The RNA attaches to the column, and the flow-throughs containing the proteins were transferred to another tube and stored in the fridge for later precipitation. The AllPrep RNA spin columns were washed by adding 700 µl Buffer RWI, centrifuging at 10000 rpm for 15 seconds, adding 500 µl Buffer RPE and centrifuging at 10000 rpm for 15 seconds. The Buffer RPE was added a second time, and the samples were centrifuged at 10000 rpm for 2 minutes. The flow-throughs between each washing-step were gently discarded, to prevent contamination of the samples. After the washing-steps, the columns were transferred to new tubes and centrifuged at full speed for 1 minute to remove all left-over liquid. The columns were transferred to new vials, and the RNA was eluted by adding 50 µl dH_2O to each column and centrifuging at 10000 for 1 minute. RNA originating from the same flask were collected in the same vial and stored at -80 °C. To avoid thawing the samples unnecessarily, 5 µl RNA was kept in a separate vial to measure the RNA content by NanoDrop.

To precipitate the proteins, 1 mL Buffer APP was added to each of the flow-throughs after the AllPrep RNA spin columns. The vials were incubated in room temperature for 10 minutes and then centrifuged at full speed for another 10 minutes. The pellets were washed by adding 500 µl 70 % ethanol followed by mixing and centrifugation at full speed for 1 minute. After washing, the pellets were carefully dried by decant the supernatant, centrifuging one more time at full speed for 1 minute and removing the excess liquid with a pipette. The pellets were dried for maximum 5 minutes in room temperature. Due to the proteomic protocol (described below), the protein pellet was resolved in 100 µl Modified reswell solution (Appendix 2) after the drying step and not the buffer ALO the Allprep® DNA/RNA/Protein Mini Kit provides. The pellets were incubated with Modified reswell solution while purifying the DNA, to completely dissolve the pellet. The dissolved pellets from the same flask were collected in the same vial and were stored at -80 °C. For quantification, 5 µl of the dissociated protein were kept in a separate vial to avoid thawing the samples unnecessarily.

The AllPrep DNA spin columns were washed by adding 500 µl Buffer AWI, centrifuged at 10000 rpm for 15 seconds, and then 500 µl Buffer AW2 followed by centrifuging at full speed for 2 minutes. The flow-throughs were discarded between the washing-steps. The DNA was eluted with 100 µl preheated EB buffer supplied by the Allprep® DNA/RNA/Protein Mini Kit for each vial. DNA originating from the same flask were

collected in the same vial and stored at -80 °C. To avoid thawing the samples unnecessarily, 5 µl DNA was kept in a separate vial to measure the DNA content by NanoDrop.

3.6 Protein quantification

The protein content was measured by using RC DC Protein Assay. Standards were made from the stock solution of bovine serum albumin from the kit. The dilutions are shown in Appendix 3. Quality controls were made from BSA in two concentrations, one in the higher range, 1.38 mg/ml, and one in the lower range, 0.376 mg/ml, of the standard curve, and diluted in dH_2O. Due to expected high concentrations, the samples were diluted 1:20 in dH_2O before the assay. The manufacturer's Microfuge Tube Assay Protocol was followed. The standards, controls and samples were firstly precipitated by adding 25 µl sample and 125 µl Reagent I to each vial. After incubating the vials at room temperature for 1 minute, 125 µl Reagent II was added. The vials were vortexed to mix the samples after adding the regents. To gather the proteins in pellets, the vials were centrifuged at full speed for 5 minutes. The pellets were dried and dissolved in 127 µl Reagent AS, which was made by adding 5 µl solution S to 250 µl solution A. The vials were incubated at room temperature for 5 minutes, or until the pellets were resolved. Finally, 1 ml Reagent B was added to each vial, and incubated in room-temperature for at least 15 minutes at room temperature to promote a color reaction in the vials. The color is stabile for 1-2 hours after adding Reagent B. The absorbance was read at 750 nm by a UV/VIS spectrophotometer. After reading the absorbance, a standard curve was made, and the protein concentration in each sample was calculated. The standard curves and the calculations of the concentrations are shown in Appendix 4.

3.7 Endocrine effects – hormone quantification

To measure hormone concentrations in thawed, collected medium, solid phase radioimmunoassay (RIA) kits were used. Standards were made by diluting the hormone for each hormone assay in cell culture medium. All the standards, controls and samples were duplicated and an average was calculated for each sample.

3.7.1 Estradiol

To measure estradiol concentrations, Coat-A-Count Estradiol kit, was used. Of each of the thawed medium samples, standards and controls, 100 µl was added to coated tubes provided by the kit. Directly after this, 1 ml of ^{125}I Estradiol was added to each vial. The samples were vortexed and incubated at room temperature for 3 h. After incubation, the tubes were decanted thoroughly and left draining for 5-10 minutes. The vials were counted for 1 minute in a gamma counter.

The standard-curve consisted of 6 standards and a reference, with the concentrations as follows: reagent blank, medium blank, 10 pg/ml, 40 pg/ml, 100 pg/ml, 250 pg/ml, 1000 pg/ml and 4000 pg/ml. The sensitivity of the assay was 10 pg/ml and the interassay variation coefficient was 10.02 %.

3.7.2 Testosterone

To measure testosterone concentrations, Coat-A-Count Total Testosterone kit, was used. Of each of the thawed medium samples, standards and controls, 50 µl was added to coated tubes provided by the kit. Directly after this, 1 ml of ^{125}I Total Testosterone was added to each vial. The samples were vortexed and incubated at 37 °C for 3 h. After incubation, the tubes were decanted thoroughly and left draining for 5-10 minutes. The vials were counted for 1 minute in a gamma counter.

The standard-curve consisted of 6 standards and a reference, with the concentrations as follows: reagent blank, medium blank, 0.1 ng/ml, 0.5 ng/ml, 2.5 ng/ml, 10 ng/ml and 20 ng/ml. The sensitivity of the assay was 0.08 ng/ml and the interassay variation coefficient was 7.08 %.

3.7.3 Progesterone

To measure progesterone concentrations, Spectria Progesterone RIA kit, was used. Of each of the thawed medium samples, standards and controls, 50 µl was added to coated tubes provided by the kit. Directly after this, 500 µl prediluted tracer was added to each vial. The samples were vortexed and incubated at room temperature for 2 h. After incubation, the tubes were decanted thoroughly and left draining for 5-10 minutes. The vials were counted for 1 minute in a gamma counter.

The standard-curve consisted of 6 standards and a reference, with the concentrations as follows: reagent blank, medium blank, 0.5 ng/ml, 1.5 ng/ml, 4 ng/ml, 10 ng/ml and 40 ng/ml. Because of an error in making the standards, a standard-curve from a previously run was used. This does not give exact amounts of progesterone in the samples, but it will give an indication if there are any differences in levels in the different exposures.

3.7.4 Cortisol

To measure cortisol concentrations, Coat-A-Count Cortisol kit, was used. Of each of the thawed medium samples, standards and controls, 25 µl was added to coated tubes provided by the kit. Directly after this, 1 ml of ^{125}I Cortisol was added to each vial. The samples were vortexed and incubated at 37 °C for 45 minutes. After incubation, the tubes were decanted thoroughly and left draining for 5-10 minutes. The vials were counted for 1 minute in a gamma counter.

The standard-curve consisted of 6 standards and a reference, with the concentrations as follows: reagent blank, medium blank, 10 ng/ml, 50 ng/ml, 100 ng/ml, 200 ng/ml and 500 ng/ml. The sensitivity of the assay was 2.5 ng/ml and the interassay variation coefficient was 9.99 %.

3.8 Sample preparations methods for 2D electrophoresis and 1D electrophoresis

The protein samples from each group were pooled to reach a final amount of proteins of 800 µg. Equal quantities of proteins were taken from each sample to make the pools' content representative to all the replicates, as shown in table 3.2.

Table 3.2: Overview over the amount of each protein sample isolated from exposed H295R cells used to make pools for proteomic analysis. Each replicate protein samples' concentration is shown in the second column. The volumes to be taken out from each sample, were calculated by dividing 800 on the number of samples, and divide that number on the protein samples' concentrations.

Dose	Conc (µg/µl)	V for a tot 800 µg	Tot volume (µl)	800/x
0,1 % DMSO	29,86	5,36		160,00
0,1 % DMSO	7,36	21,74		160,00
0,1 % DMSO	15,46	10,35		160,00
0,1 % DMSO	18,48	8,66		160,00
0,1 % DMSO	21,76	7,35		160,00
			53,46	
PFOS 175 µM	9,23	28,89		266,67
PFOS 175 µM	9,12	29,24		266,67
PFOS 175 µM	4,65	57,35		266,67
			115,48	
PFOS 200 µM	5,57	47,88		266,67
PFOS 200 µM	9,37	28,46		266,67
PFOS 200 µM	4,96	53,76		266,67
			130,10	
TBA 175 µM	15,35	17,37		266,67
TBA 175 µM	8,12	32,84		266,67
TBA 175 µM	15,61	17,08		266,67
			67,30	
TBA 200 µM	12,27	21,73		266,67
TBA 200 µM	16,33	16,33		266,67
TBA 200 µM	11,38	23,43		266,67
			61,50	
PFNA 175 µM	10,44	19,16		200,00
PFNA 175 µM	9,41	21,25		200,00
PFNA 175 µM	10,04	19,92		200,00
PFNA 175 µM	21,08	9,49		200,00
			69,82	
PFNA 200 µM	17,22	15,49		266,67
PFNA 200 µM	10	26,67		266,67
PFNA 200 µM	16,27	16,39		266,67
			58,54	

The pools were precipitated by using ReadyPrep^{TM} 2-D Cleanup Kit according to the manufacturer's instructions. The proteins in the pools were precipitated by adding 300 µl precipitating agent 1, followed by 15 minutes incubation on ice, and adding 300 µl precipitating agent 2. The samples were mixed well, and to form tight pellets, the samples were centrifuged at maximum speed for 5 minutes. The supernatants were removed before centrifuging a second time at maximum speed for 15-30 seconds. After centrifugation, the pellets were washed by adding 40 µl wash reagent 1 followed by

centrifugation at maximum speed for 5 minutes. The wash reagent was removed by using a pipette, and 25 µl of ultra pure water was added followed by centrifugation and discarding the supernatant. Prechilled Wash reagent 2 was added to the pellets, 1 ml wash reagent 2 added 5 µl of wash additive to each sample, and the vials were incubated at -20°C for 30 minutes. The vials were vortexed every ten minutes during the incubation time. After incubation, the samples were centrifuged at full speed for 5 minutes to form tight pellets. The supernatant was discarded, and the pellets were carefully dried in room temperature and resolved in 200 µl IPG Reswell buffer. To clarify the protein samples, the vials were centrifuge at maximum speed. To run 1D SDS-PAGE, 20 µl was taken out from each protein pool. Mixtures were made by adding 5 µl 3x Dissociation Buffer (see Appendix 5 for details) per 10 µl protein extract. The mixtures were heated at 100 °C in a heating block for 5 minutes.

3.9 One dimensional electrophoresis

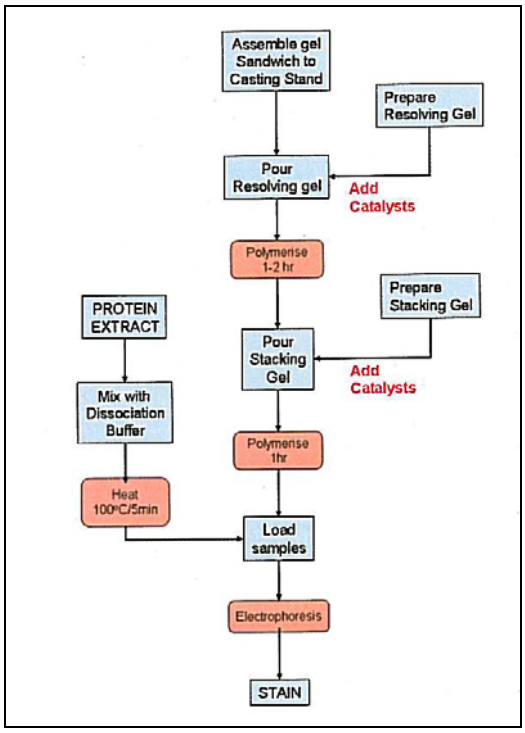


Figure 3.2: Flow chart for one dimensional gel electrophoresis. The figure is obtained from the internal manual at the Aberdeen Proteome Facility, Institute of Medical Sciences, University of Aberdeen.

The pools were analyzed by polyacrylamid slab gels to determine the amount of the protein samples from the cells to be loaded on the 2D gels. To each of the wells, 3 µl of protein mixture, described above, were added. The samples were run on duplicated gels. The gels were stained by using Colloidal Coomassie Blue G250 (Cash et al. 1995). The gels were fixed overnight in the Gel Fix solution (for description see Appendix 5) after electrophoresis. The gels were washed three times for 30 minutes each wash in MilliQ water, and equilibrated in one hour in room temperature by adding gel equilibrating solution (for description see Appendix 5) to the gels. After the equilibration, 0.2 g Coomassie Brilliant Blue G250 powder per 200 mL of gel equilibration solution was added directly to the gels. The gels were stained for up to four days, and scanned either wet or dry by using ImageScanner ^{TM} III.

Table 3.3: Overview of amount of protein-sample from exposed H295R cells added to two-dimensional gels after checking the band-strengths on a one-dimensional gel. The amount of IPG reswell buffer was adjusted according to the amount of sample added to the gel to give a final volume of 140 µl.

Sample	Amount sample added to gel (µl)	Amount IPG reswell buffer (µl)
DMSO 0,1%	20	120
TBA 175	20	120
TBA 200	18	122
PFOS 175	20	120
PFOS 200	20	120
PFNA 175	15	125
PFNA 200	18	122

3.10 Two dimensional gel electrophoresis (2D-gel)

3.10.1 First dimension

From the pooled samples, 100 µg of total protein was added to 7 cm Immobiline™ DryStrips pH 3-10 NL as described earlier by (Cash et al. 2003; Uwins et al. 2006). These DryStrips separate the proteins in the first dimension, by isoelectric pH (PI) in a non-linear range from pH 3 to 10. That means that the DryStrips can catch both the smaller and the larger proteins, but also are able to maintain a good separation of the medium

sized proteins, which are the majority. The samples were added to the IPG Reswelling Cassette, and the DryStrips were carefully lowered into the sample without creating air bubbles. The DryStrips were covered and left overnight to rehydrate, and then electrophoresed as shown in the table 3.4.

Table 3.4: The electrophoresis conditions for 7 cm Immobiline™ DryStrips pH 3-10 NL to separate the proteins in the first dimension by isoelectric pH. The current and power were held constant at 2mA and 5W, respectively.

Step	Voltage	Time
1	200 V	1 min
2	3500 V	90 min
3	3500 V	65 min

The blue color in the Reswell solution will move towards the anode, and due to this it is possible to see if the proteins are being separated. After electrophoresis, the strips can either be frozen down at -20°C or continue to the equilibration step followed by the second dimension.

The DryStrips were equilibrated 30 minutes in IPG equilibrating buffer containing 10 mg/ml DTT on a rotary shaker, and 30 minutes in IPG equilibrating buffer containing 25 mg/ml iodoacetamide. The DryStrips were drained and rinsed in MilliQ water, and loaded to the second dimension.

3.10.2 Second dimension

For the separation by mass in the second dimension, precasted commercial gels, ZOOM® Gels were used. The electrophoresis running buffer was prepared by adding MOPS 20X concentrate buffer to MilliQ water (described in Appendix 5). The inner running buffer was added antioxidant to trigger the electrophoresis reaction. The precasted gel was taken out of the protective bag, the comb was removed, and the gel and and the wells were rinsed with MilliQ water followed by a brief rinse by the running buffer. The gel was assembled with a belonging tank which was filled with running buffer and placed on an electric stirrer. The IPG strips were loaded to the gels, one strip per gel. On each gel,

there is a side track where a molecular weight marker (SeeBlue Plus 2) was added to the gel. The running conditions used were as follows:

- Step 1: Time 30 minutes, voltage 100V, current 35 mA and power 5W
- Step 2: Time 1:15 hour, voltage 200V, current 35 mA and power 5W

The molecular weight marker was used as an indicator on how electrophoresis run was proceeding. At the end of the electrophoresis, the gels were taken out of the chamber. The corners were cut in a pattern to be recognized later on, and transferred to a tray containing a gel fixer on a shaker. Four replicate gels were run for each pool of proteins.

3.11 Post electrophorestic detection of proteins

Proteins were detected by staining the gels with Colloidal Coomassie Blue G250 as described above under the description of one dimensional electrophoresis.

3.12 Gel imaging

The gels were scanned wet by using ImageScanner ^{TM} III. The scanner runs from the LabScan software. The parameters were set like in the table 3.5.

Table 3.5: Parameters set in LabScan software connected to the ImageScanner ^{TM} III when scanning the 2D-gels.

Mode	Transparent
Resolution	600 dpi
Color	Green filter

The gels were dried between to sheets of cellophane for long term storage by using EasyBreeze Gel Drier.

3.13 Spot analysis

The images were transferred to Progenesis Samespots v 3.3. This software were used to detect and quantify proteins spots, expressed as normalized volumes. The software was in addition used to match the profiles across the gel series. The image below (Fig 3.3) shows the gel used as a reference gel in the experiment. It was chosen because it was the clearest image.

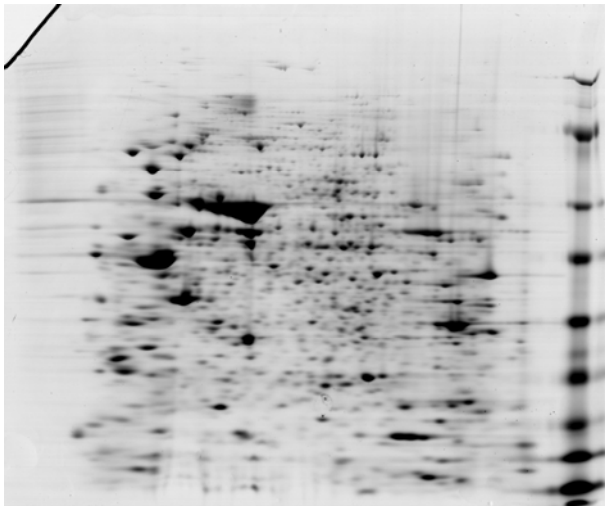


Figure 3.3: The proteomic profile of the H295R cell line. On the right, there is a molecular weight marker to be able to determine the size of the proteins. The pH ranges non-linear from 3-10 from left to right. This gel was used as a reference gel in the experiment.

Progenesis Samespots v 3.3 calculated p-values and fold change. These were used to choose spots to be further analyzed. Spots with $p \leq 0.05$ and a fold change>2 were identified by using 2D-gels and LC-MS/MS. The spot volumes were also analyzed by using JMP8 software, since the fold change values calculated from the Progenesis Samespot v 3.3 software are between the two groups with the largest difference and not necessarily between exposed groups and control. The calculated values from the Progenesis Samespot v 3.3 software are shown in the table 3.6.

Table 3.6: Calculated Anova (p-values) and fold change calculated by Progenesis software. The spots are ranked based on Anova (p-value), with the lowest p-value at the top.

Spot number	Anova (p)	Fold	Average normalized volumes						
			DMSO	PFNA 175	PFNA 200	PFOS 175	PFOS 200	TBA 175	TBA 200
949	1,780E-13	3,1	5,743E+06	1,121E+07	1,355E+07	9,987E+06	1,203E+07	4,417E+06	4,958E+06
501	1,158E-10	3,1	7,962E+06	7,092E+06	5,867E+06	3,519E+06	2,985E+06	8,723E+06	9,314E+06
1033	3,777E-10	3,9	3,678E+06	2,141E+06	1,759E+06	6,897E+06	5,293E+06	2,986E+06	3,979E+06
1277	1,167E-08	2,2	7,904E+06	1,241E+07	1,255E+07	1,216E+07	1,367E+07	6,930E+06	6,254E+06
897	9,527E-08	2,1	5,590E+06	1,175E+07	9,708E+06	9,966E+06	1,155E+07	5,659E+06	5,603E+06
974	1,747E-07	3,2	2,781E+06	1,956E+06	1,520E+06	4,666E+06	4,868E+06	2,632E+06	3,973E+06
972	3,346E-07	2,9	1,620E+06	2,868E+06	4,240E+06	2,497E+06	2,139E+06	1,450E+06	1,815E+06
896	1,713E-06	2,1	4,897E+06	8,725E+06	8,564E+06	8,618E+06	9,261E+06	4,348E+09	4,403E+06
1502	5,800E-06	2,2	1,513E+07	1,735E+07	2,226E+07	1,747E+07	1,586E+07	1,488E+07	1,022E+07
953	1,244E-05	2,4	4,314E+06	6,274E+06	6,428E+06	5,622E+06	6,871E+06	2,816E+06	3,260E+06
496	2,107E-05	2,0	3,961E+06	3,578E+06	2,824E+06	2,557E+06	2,283E+06	4,402E+06	4,491E+06
1288	2,274E-05	2,0	4,339E+06	6,117E+06	6,040E+06	7,503E+06	8,849E+06	4,770E+06	4,460E+06
523	2,790E-05	2,0	1,398E+06	1,197E+06	9,493E+05	7,297E+05	7,673E+05	1,419E+06	1,443E+06
1323	1,022E-04	2,3	3,759E+06	5,839E+06	4,238E+06	4,850E+06	4,347E+06	4,613E+06	2,496E+06
1208	2,005E-04	2,7	4,583E+06	3,932E+06	2,455E+06	1,973E+06	1,714E+06	4,236E+06	3,270E+06
853	2,206E-04	2,5	8,602E+06	9,871E+06	4,584E+06	1,104E+07	1,149E+07	9,962E+06	9,778E+06
644	1,100E-02	2,0	5,448E+06	6,922E+06	6,860E+06	7,500E+06	8,346E+06	4,170E+06	6,203E+06

The normalized volumes were the basis for electing which spots to excise. In addition each spot was assessed by looking at quality of the spots. This was done to see whether the characterization as a spot done by the software was real, or if there was contamination of ultrafine particles present. The quality was considered as good if the spot had a distinct peak, and if it was limited to a smaller area and not forming streaks.

3.14 Gel digestion

Following 2-D gel electrophoresis, proteins were visualised using colloidal Coomassie Brilliant Blue G-250 staining (as mentioned above) and the protein spots of interest excised from the gel. Proteins in the gel pieces were digested with trypsin using an Investigator ProGest robotic workstation. Briefly, proteins were reduced with DTT (60°C, 20 min), S-alkylated with Iodoacetamide (25°C, 10 min) and then digested with trypsin (37°C, 8 h). The resulting tryptic peptide extract was dried by rotary evaporation and dissolved in 0.1% formic acid for LC-MS/MS analysis.

This procedure was done by Liz Stewart at Aberdeen Proteomics, Institute of Medical Science, University of Aberdeen. The buffers and solutions used in this procedure are listed in Appendix 6.

3.15 Liquid Chromatography – Tandem Mass Spectrometry (LC-MS/MS)

Peptide solutions were analysed using an HCTultra PTM Discovery System coupled to an UltiMate 3000 LC System. Peptides were separated on a Monolithic Capillary Column (200 µm i.d. x 5 cm; Dionex part no. 161409)

Eluent A was 3% acetonitrile in water containing 0.05% formic acid, Eluent B - 80% acetonitrile in water containing 0.04% formic acid with a gradient of 3% - 45% B in 12 minutes at a flow rate of 2.5µL/min.

Peptide fragment mass spectra were acquired in data-dependent AutoMS(2) mode with a scan range of 300-1500 m/z, 3 averages, and up to 3 precursor ions selected from the MS scan 100-2200 m/z). Precursors were actively excluded within a 1.0 min window, and all singly charged ions were excluded.

This procedure was done by Liz Stewart at Aberdeen Proteomics, Institute of Medical Science, University of Aberdeen. The buffers and solutions used in this procedure are listed in Appendix 6.

3.16 Search for proteins

Peptide peaks were detected and deconvoluted automatically using Data Analysis software. Mass lists in the form of Mascot Generic Files were created automatically and used as the input for Mascot MS/MS Ions searches of the NCBInr database using the Matrix Science web server (www.matrixscience.com). The default search parameters used were: Enzyme = Trypsin, Max. Missed cleavages = 1; Fixed modifications = Carbamidomethyl (C); Variable modifications = Oxidation (M); Peptide tolerance ± 1.5 Da; MS/MS tolerance ± 0.5 Da; Peptide charge = 2+ and 3+; Instrument = ESI-TRAP. The proteins from the final report were checked by looking at the molecular weight and isoelectric pH (pI) calculated by the LC/MS-MS compared to the images of the 2D-gels. Only proteins showing good agreement with mass and pI on the 2D-gels were considered to be positive identifications (Fowler et al. 2008). In addition to this, the MOWSE scores (Pappin et al. 1993) were compared. Primarily one protein was chosen for each spot, but

in some cases there were several plausible hits. In those cases more than one protein was recognized as significant hits. In addition to using MASCOT, the proteins were also identified by using the database UniProt (www.uniprot.org).

The chosen spots were analyzed further by LC-MS/MS, and the final report contained a list of proteins based on search on the database MASCOT (http://www.matrixscience.com).

The first part of this search was done by Liz Stewart at Aberdeen Proteomics, Institute of Medical Science, University of Aberdeen.

3.17 1D gels and Western blot

The protein pools were electrophoresed in triplicates in a random order by adding 30µg protein/lane on 26-lane one-dimensional NuPage® Novex Midi Bis-Tris Gel under reducing conditions and transferred to immobilon-FL membrane. Odyssey® Two-Color Protein Molecular Weight Marker was electrophoresed in the first lane and after ten protein samples of every gel. After transfer, the membranes were treated with Odyssey Blocking Buffer diluted 1:1 with PBS buffer (for details, see Appendix 7) for one hour on rocker. The primary antibodies, StAR, vimentin, BAX, CYP17 and heat shock protein 70, were diluted in blocking buffer diluted 1:1 in PBST buffer (for details, see Appendix 7). Table 3.7 shows an overview over the dilutions of the antibodies and the manufactures. The membranes were incubated overnight on shaker in fridge. After the incubation period, the primary antibodies were collected and the membranes were washed 4 x5 minutes at RT with PBST on rocker. The secondary antibodies, were diluted in Odyssey Blocking Buffer diluted 1:1 with PBST + 0.01% SDS. The membranes were incubated in 60 minutes in RT on rocker. After incubation, the secondary antibodies were collected and the membranes were washed 4 x 5 minutes with PBST and 4 x 5 minutes with PBS.

The protein bands were visualized by using an Odyssey infrared fluorescence imager. The electronic images were analyzed using Phoretic-1D Advanced software to determine band volumes and molecular weights.

Both β-actin and α-tubulin were tested as loading controls. There were no large differences between β-actin and α-tubulin, and β-actin was chosen as a loading control.

The volumes of β-actin were compared between the exposure groups for all the antibodies to validate the analysis.

Each membrane was used for 2-3 antibodies using different channels to separate the fluorescent signals from the different antibodies. β-actin was added to all of the membranes, but due to the reuse of the membranes, not all of the pictures of the membranes presented in Results show β-actin.

Table 3.7: The table shows information on the antibodies used in western blot, including dilutions, species molecular weight (kDa) and manufacturer.

Antibody	Dilution	Species	MW	Manufacturer
CYP17	1:5000	Rabbit	58 kDa	Peter O'Shaunessey
BAX	1:200	Rabbit	23 kDa	Santa Cruz Biotechnology, INC, UK
HSP70	1:1000	Mouse	70 kDa	abcam plc, Cambridge, UK
StAR	1:500	Rabbit	30 kDa	Douglas M. Stocco, Texas Tech University, Health Science Centre
VIM	1:200	Mouse	58 kDa	abcam plc, Cambridge, UK
β actin	1:10000	Rabbit	42 kDa	abcam plc, Cambridge, UK
β actin	1:5000	Mouse	42 kDa	abcam plc, Cambridge, UK

All the data from the Western Blot were normalized by dividing the volumes on the loading control, β-actin.

3.18 Statistics

All the statistics were done by using JMP8 software. All the data sets were tested for normal distributing by using Shapiro-Wilk W test. This test has a null hypothesis that the samples come from a normally distributed population. If the calculated p-value is >0.05, the hypothesis is accepted and the samples are normally distributed. Next, the data sets were tested for homogenous variance. This was done by Levenes test. This test assesses the equality of the variance in the data sets by testing the means of each group of samples.

Cells exposed to PFOS were compared to corresponding concentrations of the salt control TBA, while cells exposed to PFNA were compared to the solvent control, DMSO 0.1 %. In addition, the salt-control, TBA, was compared to the solvent control to exclude effects of the salt alone.

P-values < 0.05 were considered significant.

3.18.1 Viability

For cells exposed to PFOS, all the results from the Alamar Blue assay were both normally distributed and had a homogenous variance. Based on this significance was tested using ANOVA. The Tukey-Kramer honestly significant difference (HSD) test was used as a post-hoc test.

The results from cells exposed to PFNA were not normally distributed, not even after log-transformation. Due to this, Bartlett's test was used to check the variance. The data set was homogenous according to this test, and the significance was tested by using Wilcoxon/Kruskal-Wallis Tests (rank sum).

3.18.2 Endocrine effects – hormone quantification

For cells exposed to PFOS, the concentrations of all the hormones were normally distributed and had homogenous variances. Based on this, significance was tested using ANOVA and the Tukey-Kramer honestly significant difference (HSD) test.

For cells exposed to PFNA, oestradiol concentrations were both normally distributed and had equal variances. Hence ANOVA and the Tukey-Kramer HSD test were used to test significance.

The testosterone concentrations were normally distributed after log-transformation and had a homogenous variance, and ANOVA and the Tukey-Kramer test was used to test differences compared to the control. Neither the progesterone concentrations nor the cortisol concentrations were normally distributed. Progesterone had a homogenous variance, while cortisol had a non-homogenous variance according to Bartlett's test. Still, both progesterone and cortisol concentrations were tested for significance by using Wilcoxon/Kruskal-Wallis Test. Kruskal-Wallis test is used for data sets which are not normally distributed or have an equal variance, and the JMP8 Software combines Wilcoxon and Kruskal-Wallis into one test.

3.18.3 Proteins

For cells exposed to PFOS, all the normalized volumes of the protein spots were both normally distributed and had equal variances. Hence, ANOVA and Tukey-Kramer Test were used to test significance.

For cells exposed to PFNA, most of the normalized volumes for the protein spots were normally distributed and had an equal variance, and ANOVA and the Tukey-Kramer Test could be used to test significance. The normalized volumes of one of the spots (spot nr 974) had to be log-transformed to become normally distributed and to get an equal variance. This spot was tested for significance by using ANOVA and Tukey-Kramer Test on the log-transformed data-set. For two of the spots (spot nr 853 and 1033), the normalized volumes were normally distributed after log-transformation, but did not have an equal variance. These were tested for significance by using Wilcoxon/Kruskal-Wallis Test.

In addition, fold change values were calculated for each protein spot and each exposure to illustrate the exposure effects. This was done by dividing the normalized volumes from each spot on the corresponding control using an Excel sheet.

3.18.4 Western blots

For cells exposed to PFOS, all normalized volumes of the proteins (volume protein/volume loading control (β-actin)) were both normally distributed and had homogenous variances. Hence ANOVA and the Tukey-Kramer HSD Test were used to test significantly differences between the exposed cells and the control.

For cells exposed to PFNA, all normalized volumes except CYP17, were normally distributed and had homogenous variances, and significance was tested by using ANOVA and Tukey-Kramer HSD Test. Normalized volumes of CYP17, were tested for significance by using Wilcoxon/Kruskal-Wallis Test.

4. Results

4.1 Viability

The cells exposed to PFOS had significantly lower viability compared to corresponding controls in all exposure doses, 150 µM, 175 µM and 200 µM (p=0.002, p=0.004 and p=0.0086 respectively) as shown in Fig 4.1. However the three concentrations were not significantly different from each other, and did not have a significant dose-response relationship.

Cells exposed to all three doses of PFNA had no significant difference in viability compared to the control (Fig 4.2). All the exposure doses gave viability >90 %.

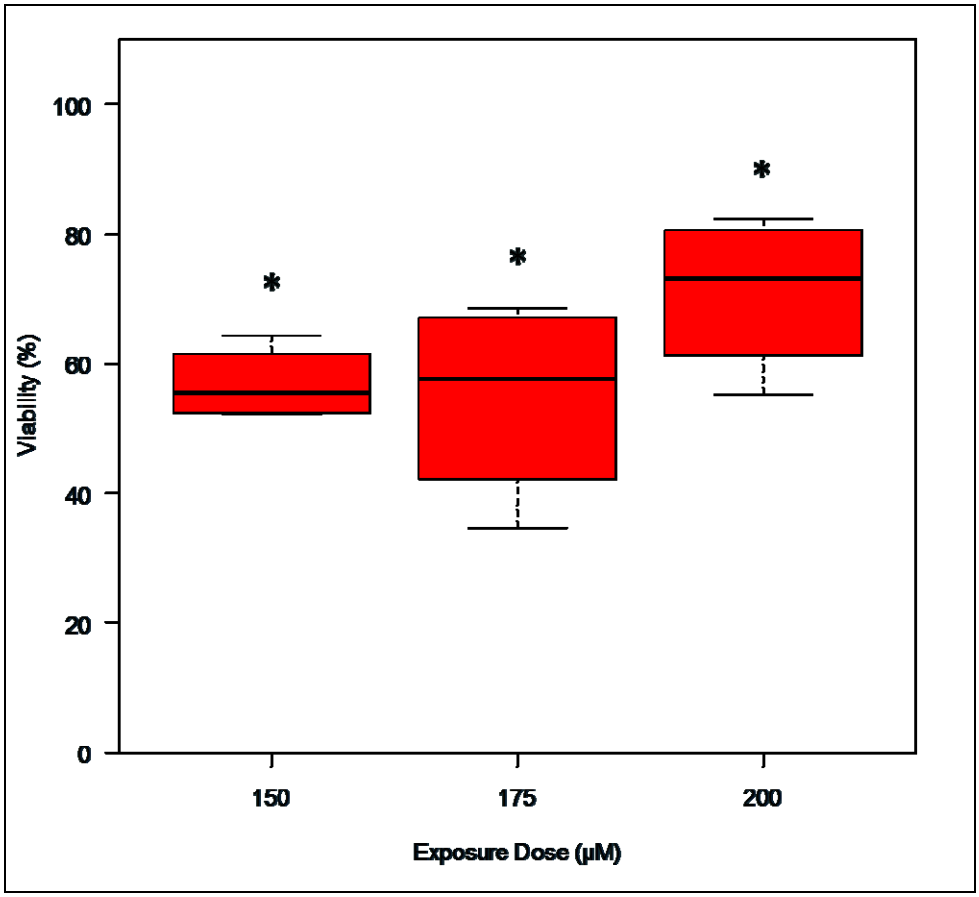


Figure 4.1: The viability of H295R cells exposed to PFOS in three different doses; 150 µM, 175 µM and 200 µM. The viability was measured by the alamarBlue® assay, and is illustrated as the % viability of the salt control, TBA, at corresponding concentrations. The boxes in the box plot contain the middle 50 % of the data (25^{th}-75^{th} percentile). The line inside the boxes indicates the median, and the whiskers indicate the maximum and the minimum data value. *: Significantly different from TBA control within exposure group (P<0.05). The significance was tested by using Tukey-Kramer HSD test in JMP8 Software.

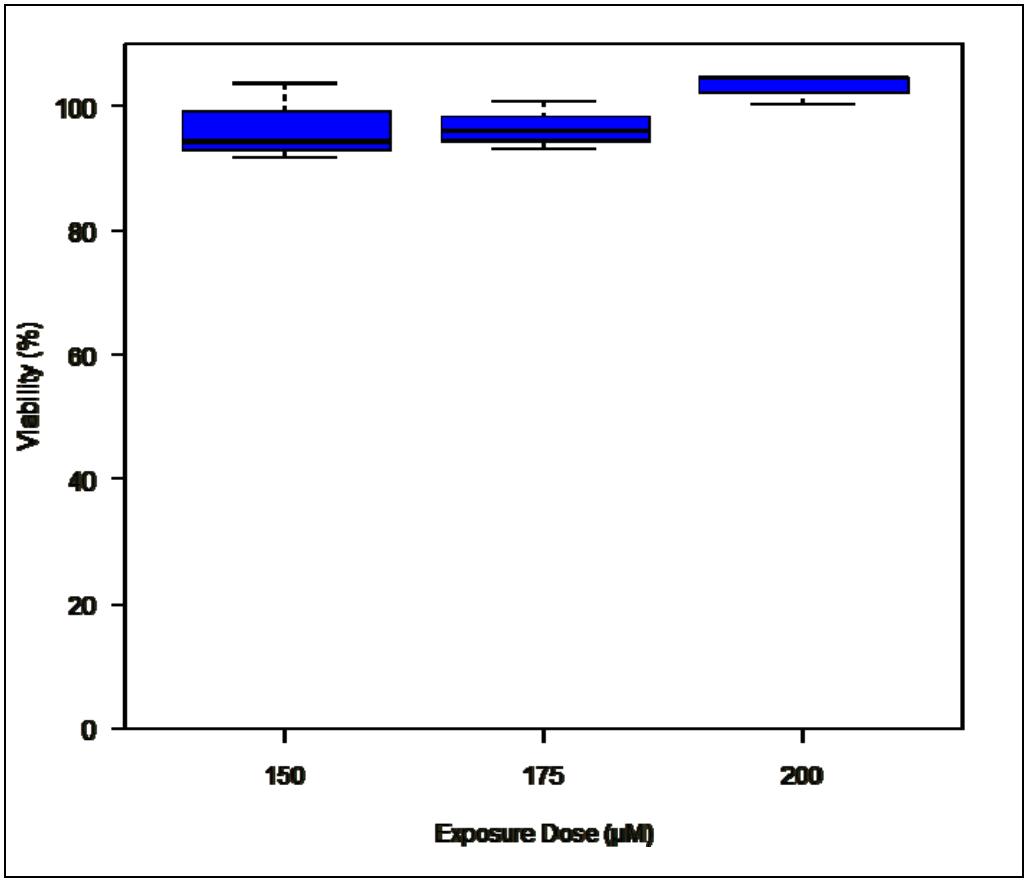


Figure 4.2: Viability of H295R cells exposed to three different doses of PFNA; 150 µM, 175 µM and 200 µM. The viability was measured by the alamarBlue® assay, and is illustrated as % viability compared to the solvent control, DMSO 0.1 %. The boxes in the box plot contain the middle 50 % of the data (25^{th}-75^{th} percentile). The line inside the boxes indicates the median, and the whiskers indicate the maximum and the minimum data value.

4.2 Endocrine effects – hormone quantification

4.2.1 Oestradiol

The highest concentration of oestradiol in medium from PFOS exposed cells was found at the lowest exposure dose (150µM) at which the mean (SE) oestradiol concentrations were 108 (8.9) pg/ml in exposed cells and 59 (7.7) pg/ml in TBA controls, respectively (P=0.0222; Fig 4.3). A significant negative dose-response relationship was found between PFOS exposure and oestradiol (p=0.0460) (Fig 4.3).

Increasing dose of PFNA gave increasing concentrations of oestradiol, but the dose-response was not significant (p=0.1278) (Fig 4.4).

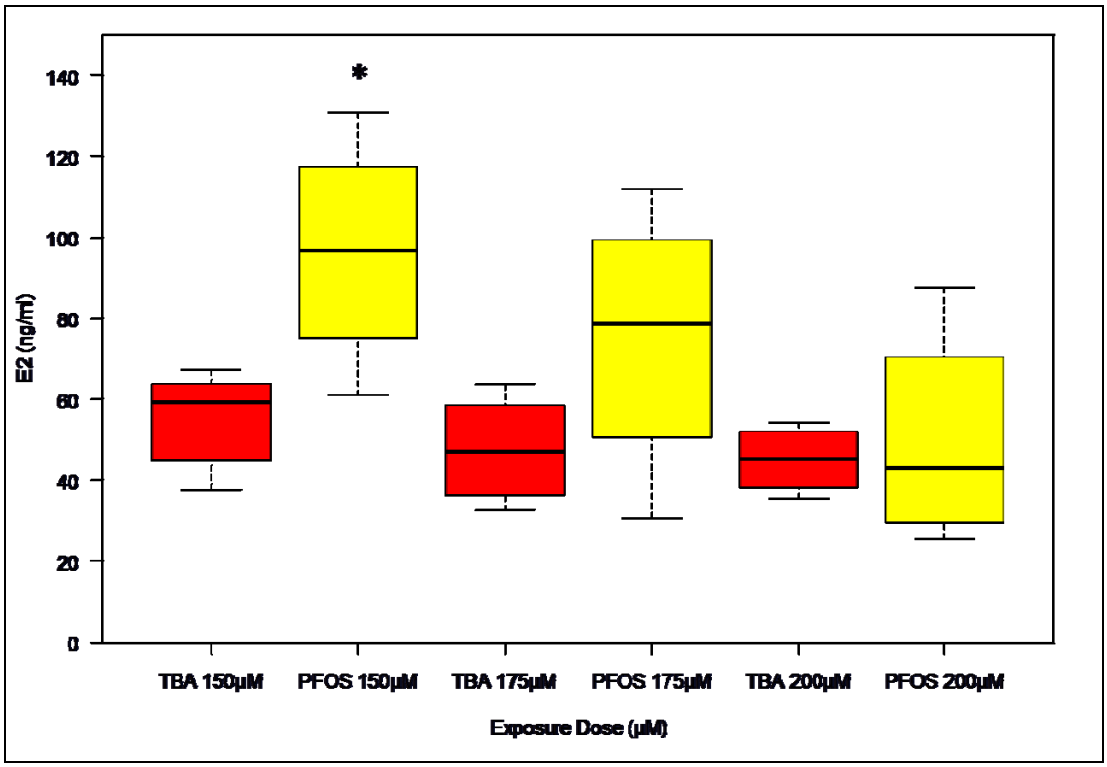


Figure 4.3: Oestradiol concentration measured in medium from H295R cells exposed to PFOS (yellow color) and in TBA controls (red color), both in three different exposure doses. TBA was used as a salt control for PFOS. The boxes in the box plot contain the middle 50 % of the data (25^{th}-75^{th} percentile). The line inside the boxes indicates the median, and the whiskers indicate the maximum and the minimum data value. *: Significantly different from TBA control within exposure group (P<0.05). The significance was tested Tukey-Kramer HSD test by using JMP8 Software.

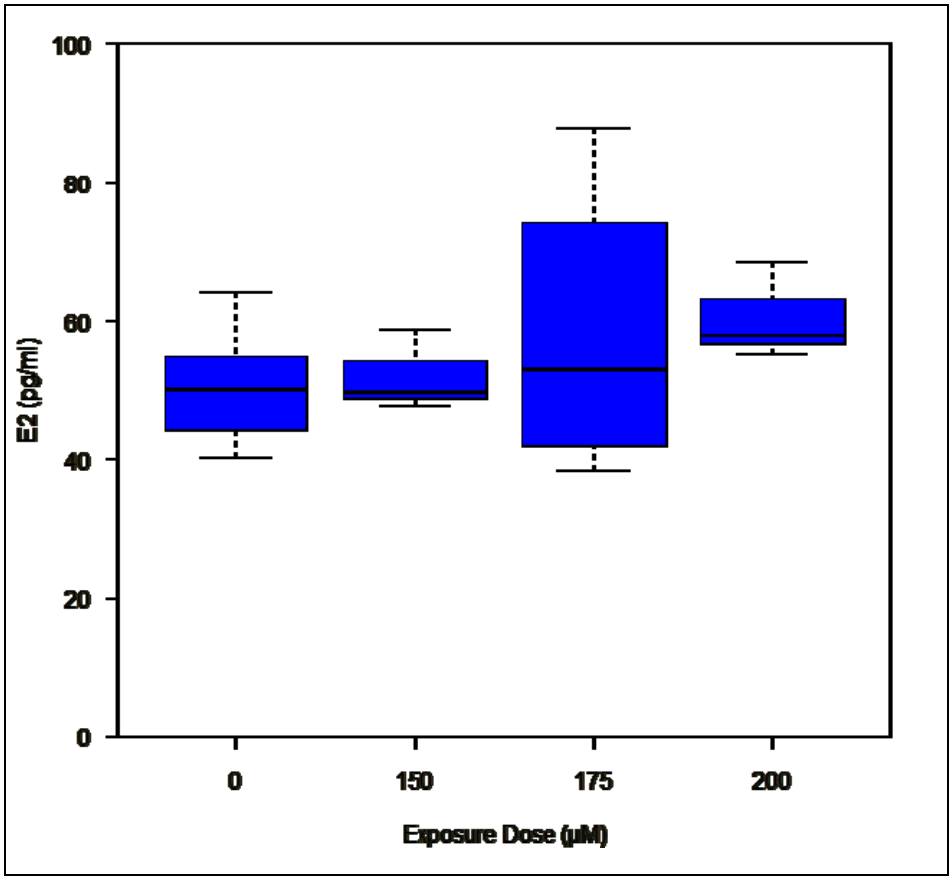


Figure 4.4: Oestradiol concentration measured in medium from H295R cells exposed to three different doses of PFNA. DMSO 0.1 % was used as a solvent control (shown as 0 in the figure). The boxes in the box plot contain the middle 50 % of the data (25^{th}-75^{th} percentile). The line inside the boxes indicates the median, and the whiskers indicate the maximum and the minimum data value.

4.2.2 Testosterone

Increasing doses of both PFOS and PFNA were associated with a significant decrease in testosterone concentration. The dose-response relationship was significant for PFNA (p=0.0021), but not for PFOS (p=0.1030) with increasing dose. The reduction in the mean testosterone at the highest dose (200 µM) was 50% and 30% compared with controls for PFOS and PFNA exposed cells, respectively (Figs 4.5 and 4.6).

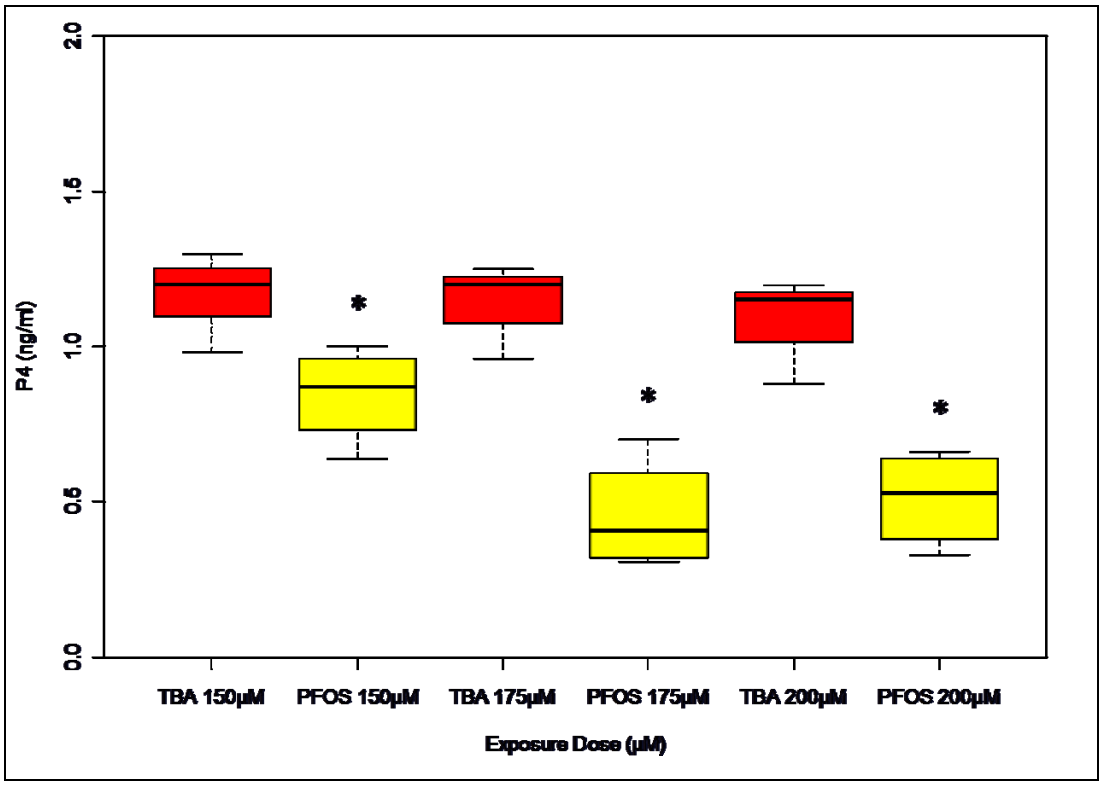


Figure 4.5: Testosterone concentration measured in medium from H295R cells exposed to PFOS (yellow color) and in TBA controls (red color), both in three different exposure doses. TBA was used as a salt control for PFOS. The boxes in the box plot contain the middle 50 % of the data (25^{th}-75^{th} percentile). The line inside the boxes indicates the median, and the whiskers indicate the maximum and the minimum data value. *: Significantly different from TBA control within exposure group (P<0.05). The significance was tested with Tukey-Kramer HSD test by using JMP8 Software.

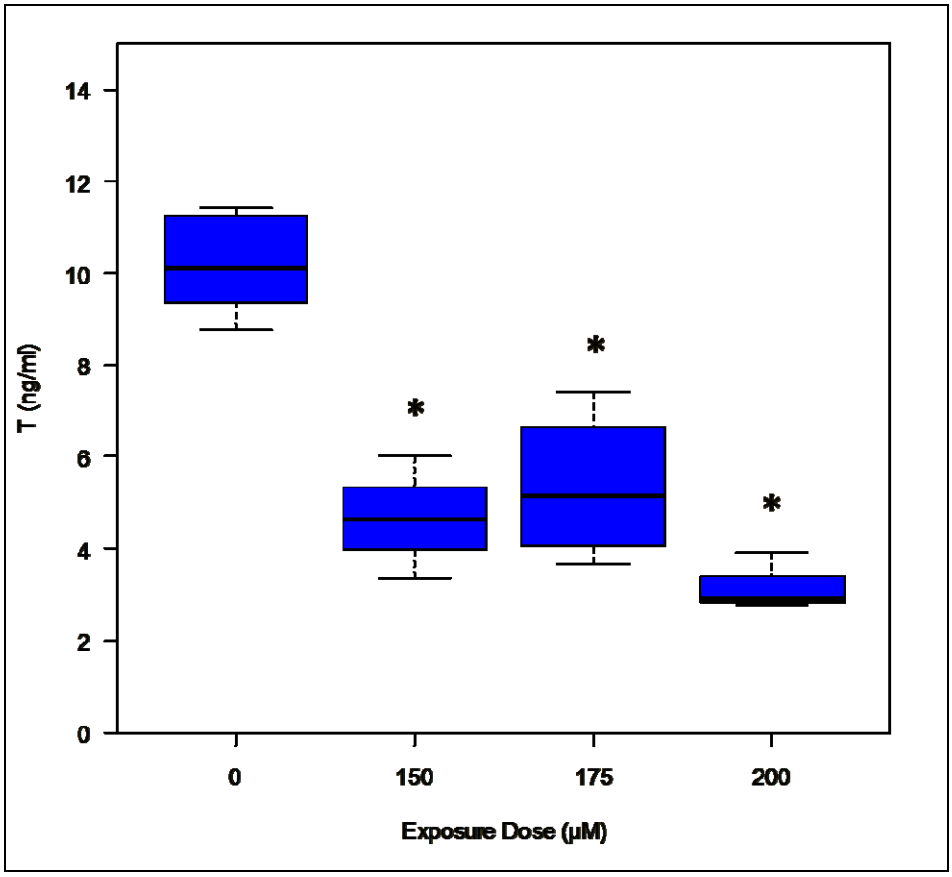


Figure 4.6: Testosterone concentration measured in medium from H295R cells exposed to three different doses of PFNA. DMSO 0.1 % was used as a solvent control (shown as 0 in the figure). The boxes in the box plot contain the middle 50 % of the data (25^{th}-75^{th} percentile). The line inside the boxes indicates the median, and the whiskers indicate the maximum and the minimum data value. *: Significantly different from DMSO 0.1 % control within exposure group (P<0.05). The significance was tested with Tukey-Kramer HSD test by using JMP8 Software.

4.2.3 Progesterone

Exposure with PFOS and PFNA both decreased production of progesterone. All exposure doses (150, 175 and 200 µM) differed significantly from controls (Figs 4.7 and 4.8). A significant negative dose response relationship was found for both compounds. The highest exposure doses of PFOS and PFNA caused a 50% and 75% reduction in mean progesterone concentration, respectively.

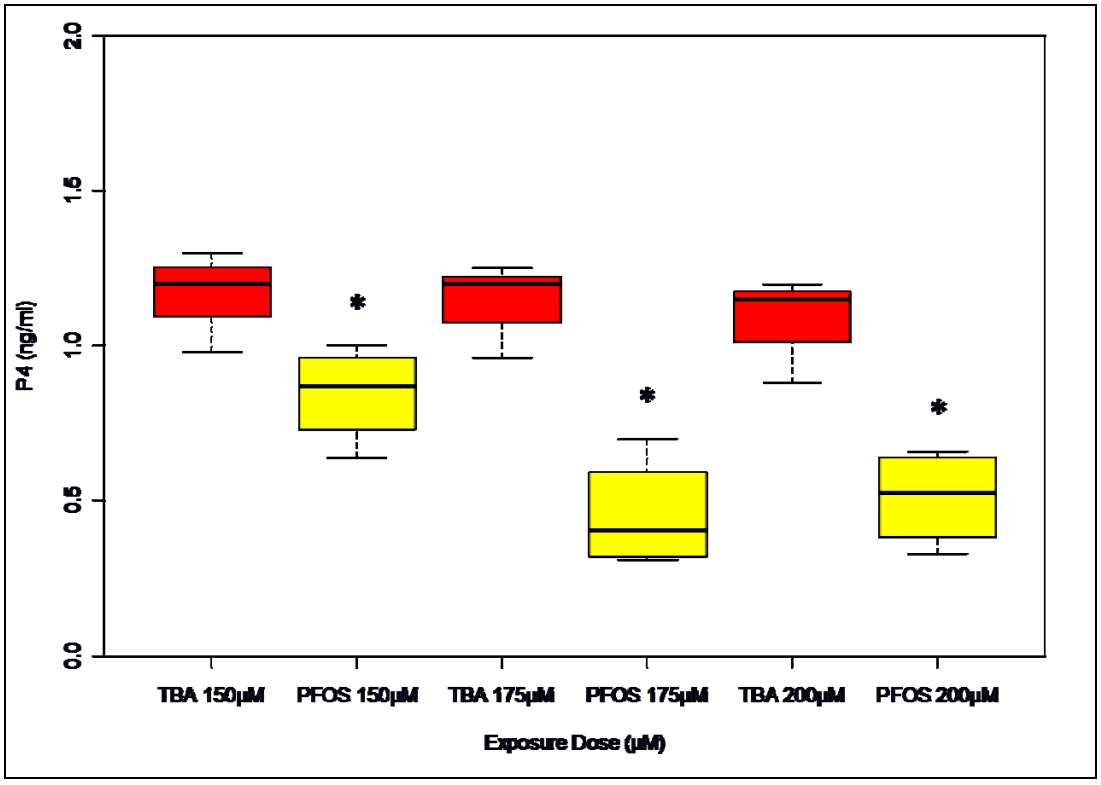


Figure 4.7: Progesterone concentration measured in medium from H295R cells exposed to PFOS (yellow color) and in TBA controls (red color), both in three different exposure doses. TBA was used as a salt control for PFOS. The boxes in the box plot contain the middle 50 % of the data (25^{th}-75^{th} percentile). The line inside the boxes indicates the median, and the whiskers indicate the maximum and the minimum data value. *: Significantly different from TBA control within exposure group (P<0.05). The significance was tested with Tukey-Kramer HSD test by using JMP8 Software.

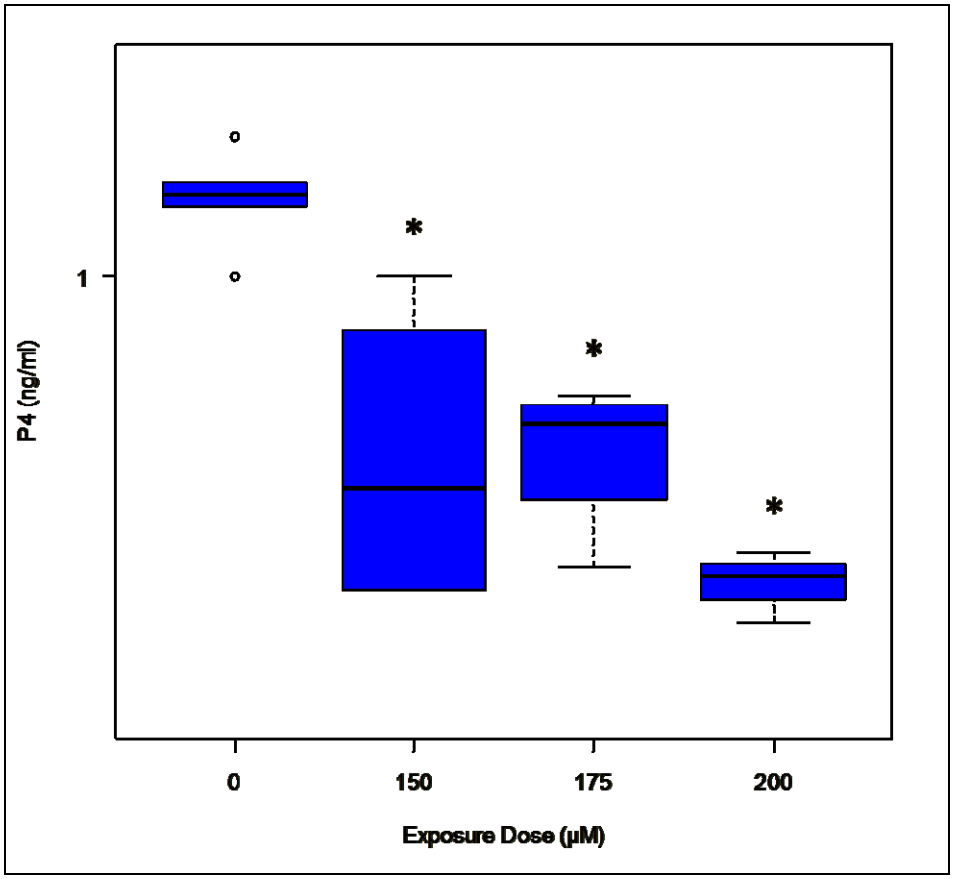


Figure 4.8: Progesterone concentration measured in medium from H295R cells exposed to three different doses of PFNA. DMSO 0.1 % was used as a solvent control (shown as 0 in the figure). The boxes in the box plot contain the middle 50 % of the data (25^{th}-75^{th} percentile). The line inside the boxes indicates the median, and the whiskers indicate the maximum and the minimum data value. The two points over and under the box representing the solvent control, are outliers. *: Significantly different from DMSO 0.1 % control within exposure group (P<0.05). The significance was tested with Wilcoxon/Kruskal-Wallis test by using JMP8 Software.

4.2.4 Cortisol

Exposure with PFOS and PFNA both caused a decrease in production of cortisol. All exposure doses (150, 175 and 200 µM) for cells exposed to PFOS and the two highest doses (175 and 200 µM) for cells exposed to PFNA differed significantly from their respective controls (Figs 4.9 and 4.10). A significant negative dose response relationship was found for both compounds. The highest exposure doses of PFOS and PFNA caused a 70% reduction and 75% reduction in mean cortisol concentration, respectively.

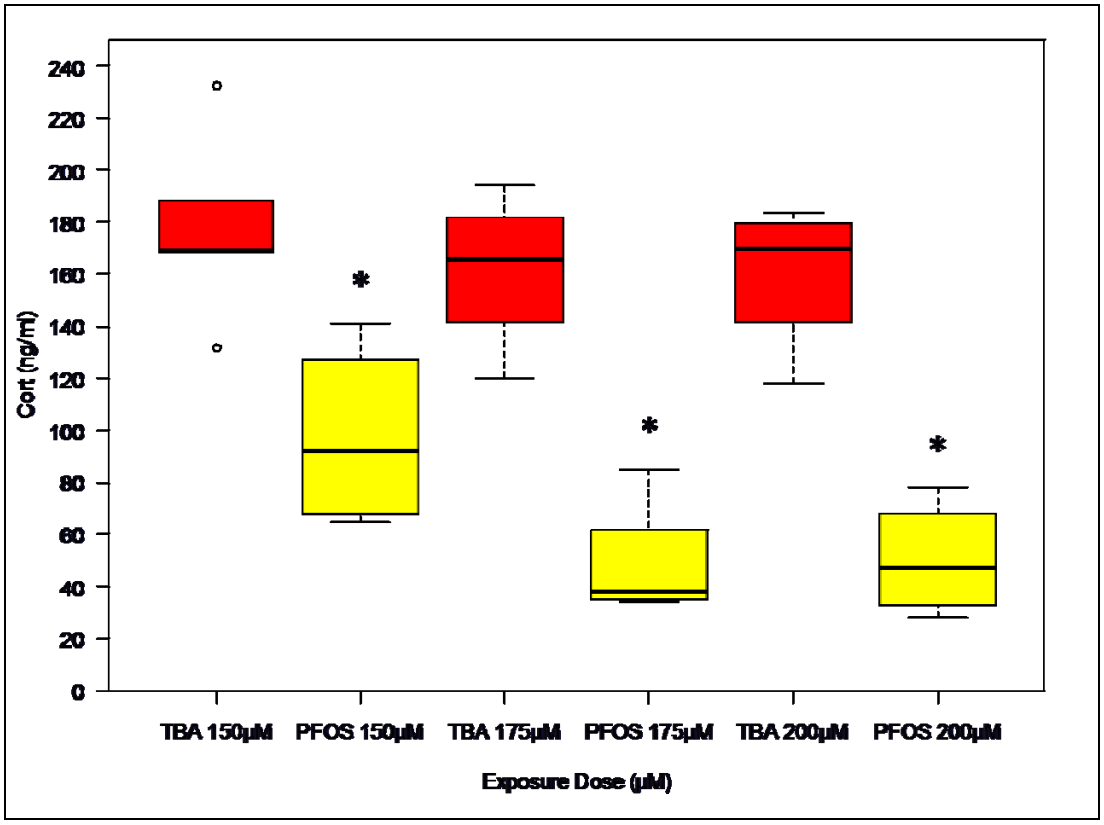


Figure 4.9: Cortisol concentration measured in medium from H295R cells exposed to PFOS (yellow color) and in TBA controls (red color), both in three different exposure doses. TBA was used as a salt control for PFOS. The boxes in the box plot contain the middle 50 % of the data (25^{th}-75^{th} percentile). The line inside the boxes indicates the median, and the whiskers indicate the maximum and the minimum data value. *: Significantly different from TBA control within exposure group (P<0.05). The significance was tested with Tukey-Kramer HSD test by using JMP8 Software.

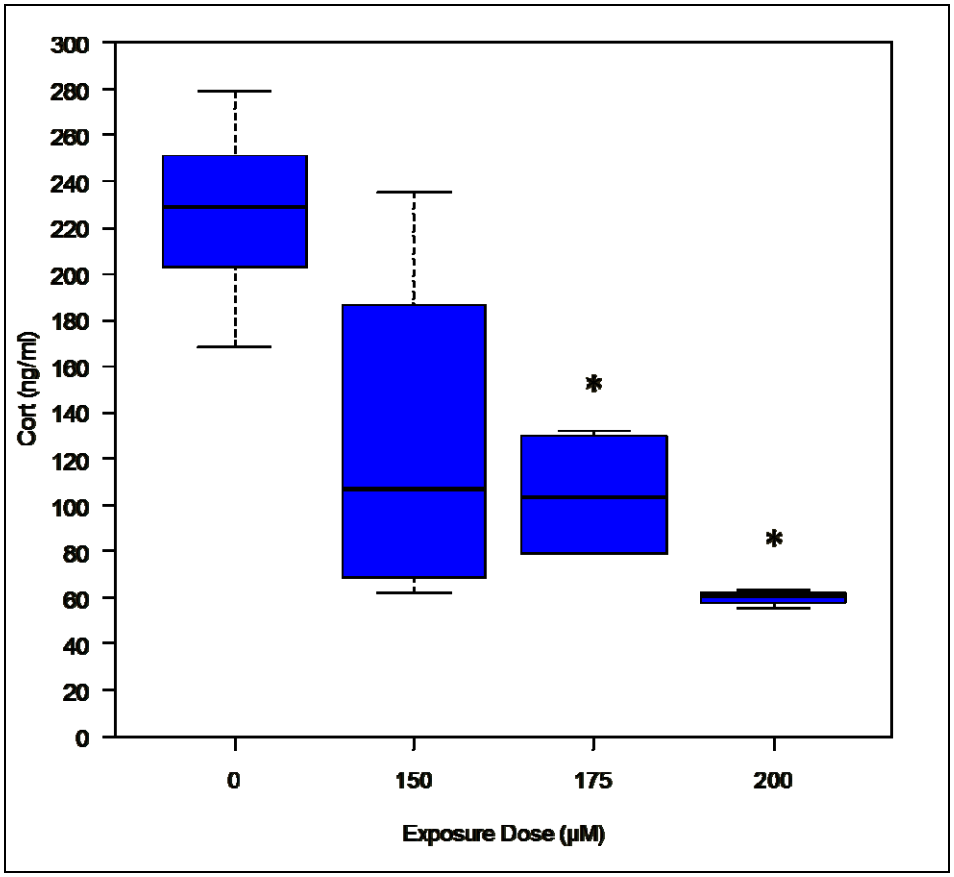


Figure 4.10: Cortisol concentration measured in medium from H295R cells exposed to three different doses of PFNA. DMSO 0.1 % was as a solvent control (shown as 0 in the figure). The boxes in the box plot contain the middle 50 % of the data (25^{th}-75^{th} percentile). The line inside the boxes indicates the median, and the whiskers indicate the maximum and the minimum data value. *: Significantly different from DMSO 0.1 % control within exposure group (P<0.05). The significance was tested Wilcoxon/Kruskal-Wallis test by using JMP8 Software.

4.3 Proteomics

4.3.1 Proteomic profile

The proteomic profile of the H295R cell line is shown in Fig. 4.11. The H295R cells gave a clean profile on the 2D-gels. Few of the gels had smears, and although some contamination of keratin, most likely from making the gels or handling the gels, the spots were clear with defined peaks. The Progenesis Samespots v3.3 defined 1409 spots. After checking each spot, 703 spots were included and 706 spots were excluded from the results. Of the 703 spots included in the results, 180 spot were statistically significant different between groups ($p<0.05$). Seventeen spots were excised to be further analyzed. An overview of the excised spots is shown in Fig 4.11 below.

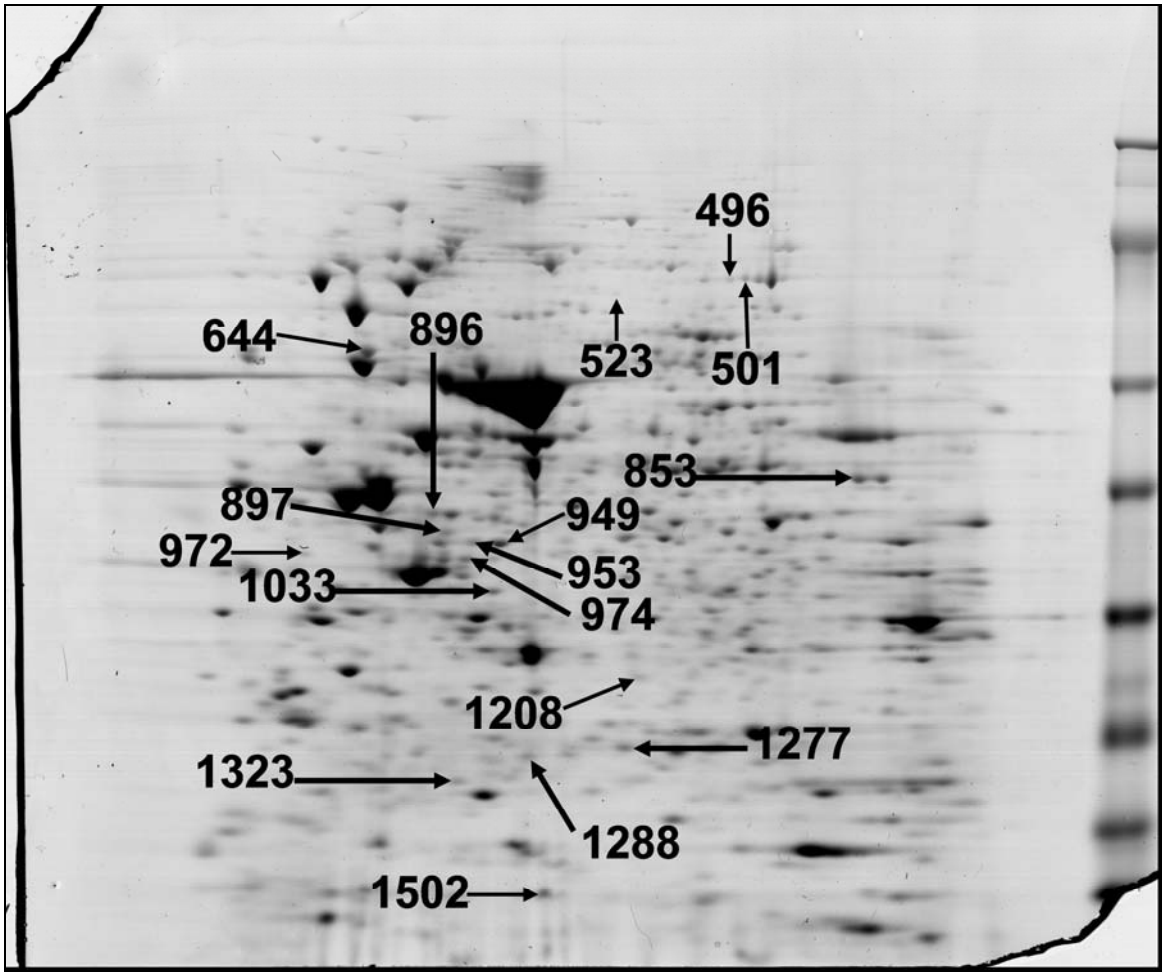


Figure 4.11: The proteomic profile of the H295R cell line. At the right in the figure, there is a molecular weight marker used to decide the molecular weight of the proteins. The pH ranges non-linear from 3-10 from left to right. Each of the numbers, with belonging arrow, represents one spot that was excised and further analyzed.

4.3.2 Identified proteins

The proteins that were identified and their level of expression (fold changes) are outlined in tables 4.1 and 4.2, respectively. Table 4.1 gives an overview over the proteins found and their functions and subcellular locations. Table 4.2 shows fold changes and p-values for the cells exposed to both concentrations of PFOS and PFNA, compared to their respective controls, TBA and DMSO. These fold changes were recalculated due to different controls used for PFOS and PFNA.

Based on comparison of listed pI and mass for each protein and the gels, spot 897 identified three proteins as possible hits and spot 644 identified two proteins as possible hits. For the rest of the spots, only the most probable proteins were taken in consideration. Spot 1323 and 1502 did not have any probable protein hits because neither of the hits matched the proteins mass and PI. These spots were probably contaminated and were excluded from the study.

Several of the proteins identified were transcription factors involved in protein synthesis, such as eukaryotic translation initiation factor 3, eukaryotic translation elongation factor 2 and eukaryotic initiation factor 4AII. In addition there was a structural protein, vimentin, a stress related protein, heat shock 70 kDa protein (HSP70) and a transport related protein, albumin, among the findings. All the proteins and their function are described in more detail in table 4.1

Table 4.1: Overview over the most significant protein findings in H295R cells exposed to PFOS and PFNA with TBA and DMSO 0.1 % as controls, respectively, identified by LC/MS-MS. The table gives information about each proteins mass (Da), isoelectric pH (PI), MOWSE score*, functions and subcellular location.

Spot	Protein	Mass(Da)	PI	MOWSE score	Gene Name	Function	Subcellular location
Transport							
949	Albumin, isoform CRA_t	60211	6,66	111	ALB	Transport	Cytoplasm
974	Serum albumin	71343	5,92	69	ALB	Transport	Cytoplasm
Transcription/protein synthesis							
1033	Eukaryotic translation initiation factor 3, subunit M	42932	5,41	56	EIF3M	Required for several steps in the initiation of the protein synthesis	Cytoplasm
897	Eukaryotic initiation factor 4AII	46593	5,33	241	EIF4A2	ATP dependent RNA helicase involved in cap recognition and required for mRNA binding to ribosome	Cytoplasm
501	Eukaryotic translation elongation factor 2	96246	6,41	700	EEF2	Promotes the GTP-dependent translocation of the nascent protein chain from A-site to P-site of ribosome	Cytoplasm
496	Eukaryotic translation elongation factor 2	96246	6,41	441	EEF2	Promotes the GTP-dependent translocation of the nascent protein chain from A-site to P-site of ribosome	Cytoplasm
953	Chain A, Chrystal structure of the Eif4a-Pdcd4 complex	44466	5,49	63	EIF4A1-DDX2A	Eif4A: ATP-dependent RNA helicase. Pdcd4: Tumor suppressor, inhibits Eif4A	Cytoplasm, Nucleus
897	Chain A, Chrystal structure of the Eif4a-Pdcd4 complex	44466	5,49	293	EIF4A1-DDX2A	Eif4A: ATP-dependent RNA helicase. Pdcd4: Tumor suppressor, inhibits Eif4A	Cytoplasm, Nucleus
1208	High mobility group protein B1 (HMGB1)	25148	5,76	177	FM1	Binds preferentially single-stranded DNA and unwinds double stranded DNA	Nucleus
523	unr protein (Cold shock domain-containing protein E1)	86599	6,17	161	CSDE1	DNA dependent regulation of transcription	Cytoplasm
897	Actin-like 6A isoform 1	47944	5,39	216	ACTL6A	Involved in transcriptional activation and repression of select genes by chromatin remodeling	Nucleus
Stress							
644	Heat shock 70kDa protein 5 (HSP70)	72402	5,07	1015	HSPA5	Involved in folding and assembly of proteins in ER. May play a role in monitoring transport of proteins in the cell	ER
644	Protein disulfide isomerase A4 precursor	73229	4,96	725	PDIA4	Catalyzes the rearrangement of -S-S- bonds in proteins	ER
Structure							
972	Vimentin (vim)	53738	5,03	399	VIM	Class-III intermediate filaments involved in cell motion and interspecies interaction between organisms	Cytoplasm
Lipid synthesis, gluconeogensis							
1277	Triosephosphate isomerase	26894	7,1	65	TPI1	Fatty acid biosynthesis, gluconeogenesis, glycolysis, lipid synthesis, pentose shunt	Cytoplasm
Cell cycle progression							
896	NEDD8-activating enzyme E1 catalytic subunit	52495	5,37	170	UBA3	Down-regulates steroid receptor activity. Necessary for cell cycle progression	Nucleus
Energy production							
853	ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	59785	9,07	1083	ATP5A1	Produces ATP from ADP Tumor modifier	Mitochondria
Purine salvage pathway							
1288	Phosphoribosyl transferase domain containing 1	66149	8,16	48	PRTFDC1	Purine ribonucleoside salvage, hypoxanthine phosphoribosyltransferase activity	Cytoplasm

*MOWSE-score in an algorithm described by (Pappin et al. 1993).

Table 4.2: Overview over fold change and p-values for all significant proteins findings for H295R cells exposed to PFOS and PFNA. Proteins from cells exposed to PFOS and PFNA have been compared to their respective controls, TBA and DMSO. P<0.05 are written in red.

Protein	Exposure	Fold change	P-value
Albumin, isoform CRA_t	PFOS 175 µM	2,3	<0,0001
	PFOS 200 µM	2,4	<0,0001
	PFNA 175 µM	1,9	0,0005
	PFNA 200 µM	2,4	0,0002
Eukaryotic translation initiation factor 3, subunit M	PFOS 175 µM	2,3	<0,0001
	PFOS 200 µM	1,3	0,0037
	PFNA 175 µM	-1,5	0,0833
	PFNA 200 µM	-2,1	0,0209
Serum albumin	PFOS 175 µM	1,8	<0,0001
	PFOS 200 µM	1,2	0,0266
	PFNA 175 µM	-1,1	0,7728
	PFNA 200 µM	-1,9	0,0209
Phosphoribosyl transferase domain containing 1	PFOS 175 µM	1,6	0,0029
	PFOS 200 µM	2,0	0,0017
	PFNA 175 µM	1,7	0,0641
	PFNA 200 µM	1,4	0,0324
Eukaryotic translation elongation factor 2	PFOS 175 µM	-2,5	<0,0001
	PFOS 200 µM	-3,1	0,0002
	PFNA 175 µM	-1,2	0,1671
	PFNA 200 µM	-1,4	0,0409
Eukaryotic translation elongation factor 2	PFOS 175 µM	-1,7	<0,0001
	PFOS 200 µM	-2,0	0,0042
	PFNA 175 µM	-1,1	0,3922
	PFNA 200 µM	-1,4	0,0358
HMG-1	PFOS 175 µM	-2,1	0,002
	PFOS 200 µM	-1,9	0,0175
	PFNA 175 µM	-1,2	0,3153
	PFNA 200 µM	-1,9	0,0188
Chain A, Chrystal structure of the Eif4a-Pdcd4 complex	PFOS 175 µM	2,0	0,0002
	PFOS 200 µM	2,1	0,0003
	PFNA 175 µM	1,6	0,0942
	PFNA 200 µM	1,5	0,1116
Triosephosphate isomerase	PFOS 175 µM	1,8	0,0014
	PFOS 200 µM	2,2	<0,0001
	PFNA 175 µM	1,7	0,0081
	PFNA 200 µM	1,6	0,0023
unr protein (Cold shock domain-containing protein E1)	PFOS 175 µM	-1,9	0,0035
	PFOS 200 µM	-1,9	0,006
	PFNA 175 µM	-1,3	0,0454
	PFNA 200 µM	-1,5	0,0134
Chain A, Chrystal structure of the Eif4a-Pdcd4 complex	PFOS 175 µM	1,8	0,0012
	PFOS 200 µM	2,1	<0,0001
	PFNA 175 µM	2,2	0,0016
	PFNA 200 µM	1,7	0,0007
Eukaryotic initiation factor 4AII	PFOS 175 µM	1,8	0,0012
	PFOS 200 µM	2,1	<0,0001
	PFNA 175 µM	2,2	0,0016
	PFNA 200 µM	1,7	0,0007

Table 4.2 continues:

Protein	Exposure	Fold change	P-value
Actin-like 6A isoform 1	PFOS 175 µM	1,8	0,0012
	PFOS 200 µM	2,1	<0,0001
	PFNA 175 µM	2,2	0,0016
	PFNA 200 µM	1,7	0,0007
NEDD8-activating enzyme E1 catalytic subunit	PFOS 175 µM	2,0	0,0012
	PFOS 200 µM	2,1	0,0016
	PFNA 175 µM	1,8	0,0127
	PFNA 200 µM	1,7	0,0095
Vimentin	PFOS 175 µM	1,7	0,007
	PFOS 200 µM	1,2	0,2437
	PFNA 175 µM	2,0	0,0045
	PFNA 200 µM	2,6	<0,0001
Heat shock 70kDa protein 5	PFOS 175 µM	1,6	0,0132
	PFOS 200 µM	2,0	0,1597
	PFNA 175 µM	1,2	0,29
	PFNA 200 µM	1,3	0,1856
Protein disulfide isomerase A4 precursor	PFOS 175 µM	1,6	0,0132
	PFOS 200 µM	2,0	0,1597
	PFNA 175 µM	1,2	0,29
	PFNA 200 µM	1,3	0,1856
ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit 1, cardiac muscle	PFOS 175 µM	1,1	0,5011
	PFOS 200 µM	1,2	0,3634
	PFNA 175 µM	1,2	0,2482
	PFNA 200 µM	-1,9	0,0209

4.3.3 TBA

Out of seventeen excised spots, six were significantly different when TBA was compared to DMSO (Table 4.3). Two of these spots had no possible protein hits, and are not shown.

Table 4.3: Overview over fold change and p-values for all significant proteins findings for H295R cells exposed TBA. The protein spots from the cells have been compared to DMSO 0.1 %. P<0.05 are written in red.

Protein	Exposure	Fold change	P-value
Albumin, isoform CRA_t	TBA 175	-1,3	0,0002
	TBA 200	-1,2	0,0071
Serum albumin	TBA 175	-1,1	0,7683
	TBA 200	1,4	0,0381
HMG-1	TBA 175	-1,1	0,5627
	TBA 200	-1,4	0,0492
Triosephosphate isomerase	TBA 175	-1,1	0,0664
	TBA 200	-1,3	0,0064

4.4 Western blots

4.4.1 CYP17

CYP17 was not significant differently expressed in PFOS exposed cell (Fig 4.13), or in PFNA exposed cells (Fig 4.14). A picture of the membrane is shown in Fig 4.12.

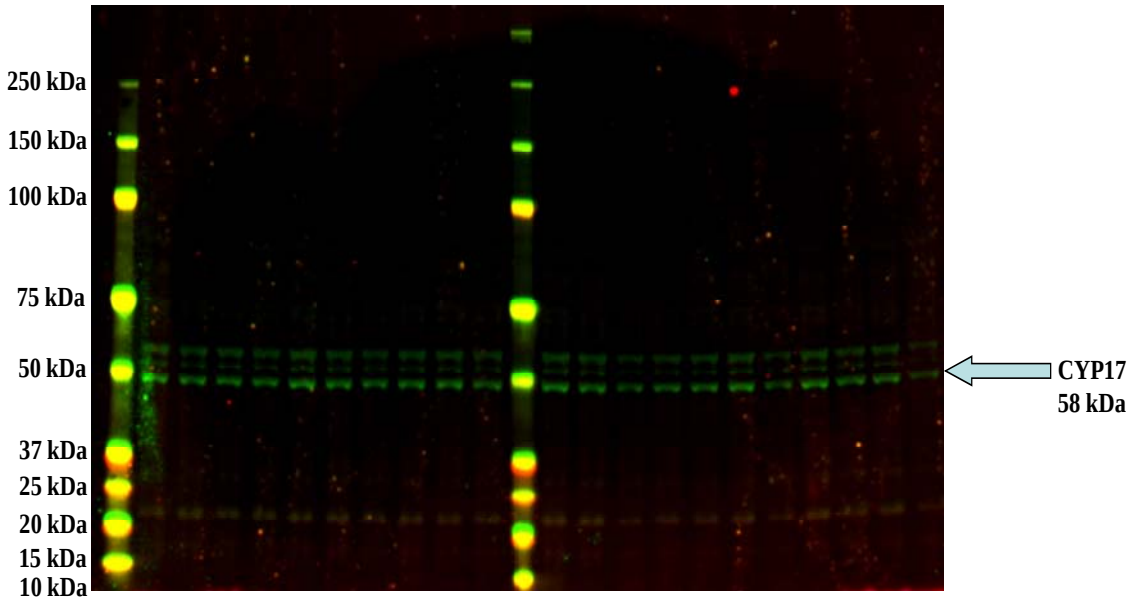


Figure 4.12: Proteins pools from H295R cells exposed to PFOS and PFNA were electrophorized and transferred to a membrane, with TBA and DMSO 0.1 % as their respective controls. The membrane was coated with antibody against CYP17 (MW 58kDa), and thereby visualized, as seen in this figure. The protein pools from the cells were run as triplicates in a random order on the membrane. In addition a molecular weight marker was added in the first lane and in the middle lane, to determine the size of the antibody (MW listed to the left). The arrow at the right points at the bands used in statistics.

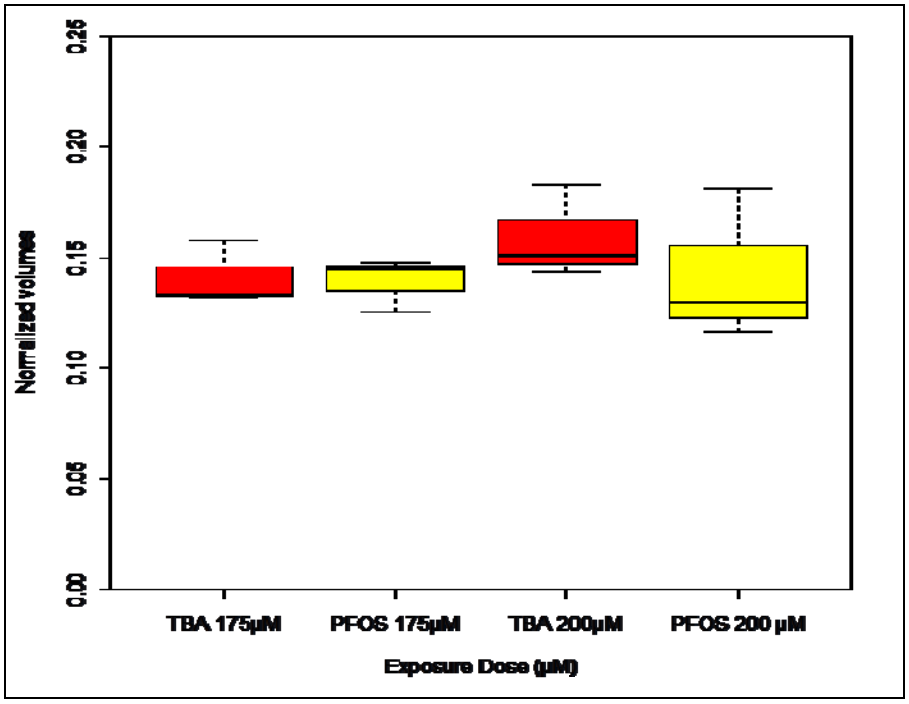


Figure 4.13: Average levels of CYP17 normalized with b-actin as loading control in H295R cells exposed to PFOS compared to corresponding concentrations of TBA. The boxes in the box plot contain the middle 50 % of the data (25th-75th percentile). The line inside the boxes indicates the median, and the whiskers indicate the maximum and the minimum data value. The red boxes represent cells exposed to TBA, while the yellow boxes represent cells exposed to PFOS.

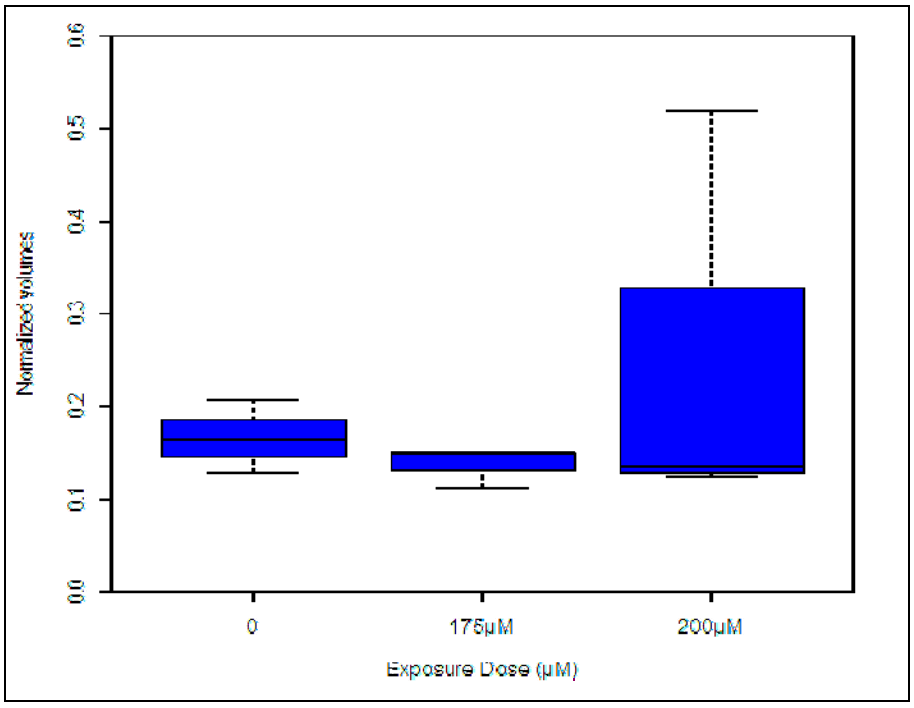


Figure 4.14: Average levels of CYP17 normalized with b-actin as loading control in H295R cells exposed to PFNA compared to DMSO 0.1 % (shown as 0 in the figure). The boxes in the box plot contain the middle 50 % of the data (25th-75th percentile). The line inside the boxes indicates the median, and the whiskers indicate the maximum and the minimum data value.

4.4.2 BAX

Exposure with PFOS (175 and 200 µM) caused a significant reduction in BAX expression compared with the controls, p=0.0091 and p=0.0074 respectively (Fig 4.16). PFNA exposure had no significant effect on BAX expression (Fig 4.17). A picture of the membrane is shown in Fig 4.15.

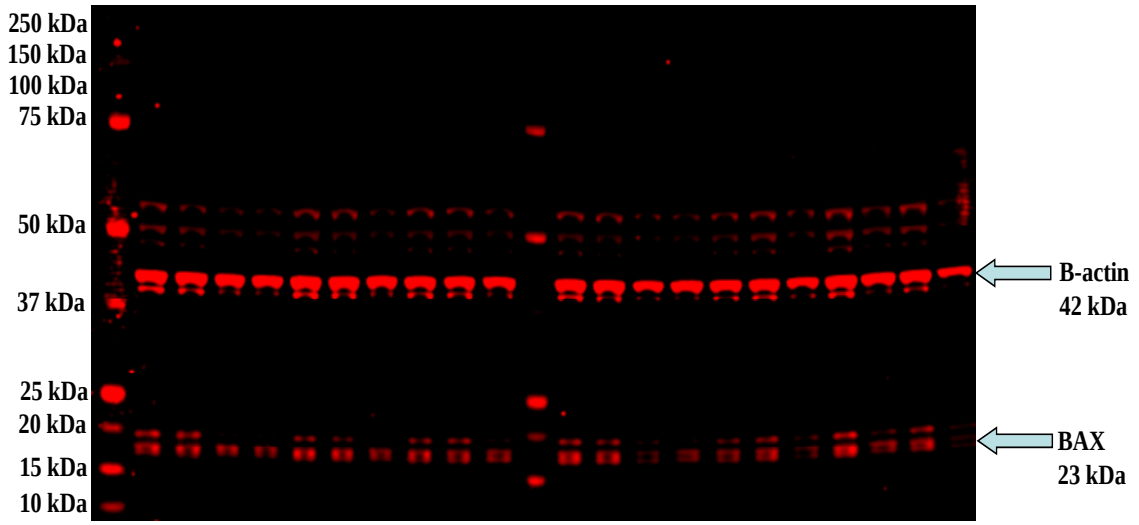


Figure 4.15: Proteins pools from H295R cells exposed to PFOS and PFNA were electrophorized and transferred to a membrane, with TBA and DMSO 0.1 % as their respective controls. The membrane was coated with antibody against BAX (MW 23 kDa) and the loading control, β-actin (42 kDa), and thereby visualized, as seen in this figure. The protein pools from the cells were run as triplicates in a random order on the membrane. In addition a molecular weight marker was added in the first lane and in the middle lane, to determine the size of the antibody (MW listed to the left). The arrows at the right point at the bands used in statistics.

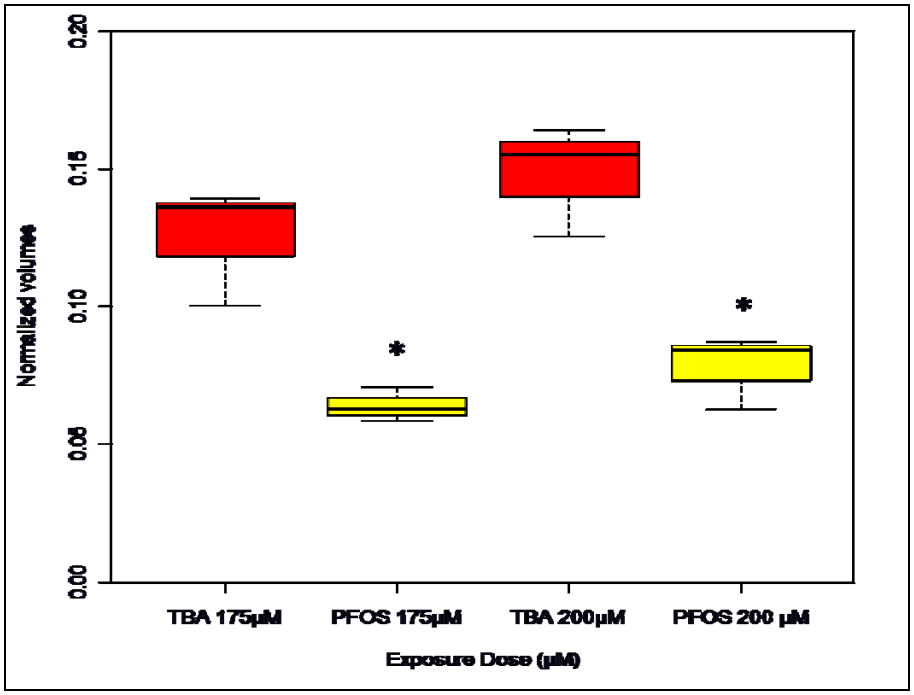


Figure 4.16: Average levels of BAX normalized with b-actin as loading control in H295R cells exposed to PFOS compared to corresponding concentrations of the control TBA. The boxes in the box plot contain the middle 50 % of the data (25^{th}-75^{th} percentile). The line inside the boxes indicates the median, and the whiskers indicate the maximum and the minimum data value. The red boxes represent cells exposed to TBA, and the red boxes represent cells exposed to PFOS. *: Significantly different from TBA control within exposure group (P<0.05). The significance was tested by using Tukey-Kramer HSD Test in JMP8 Software.

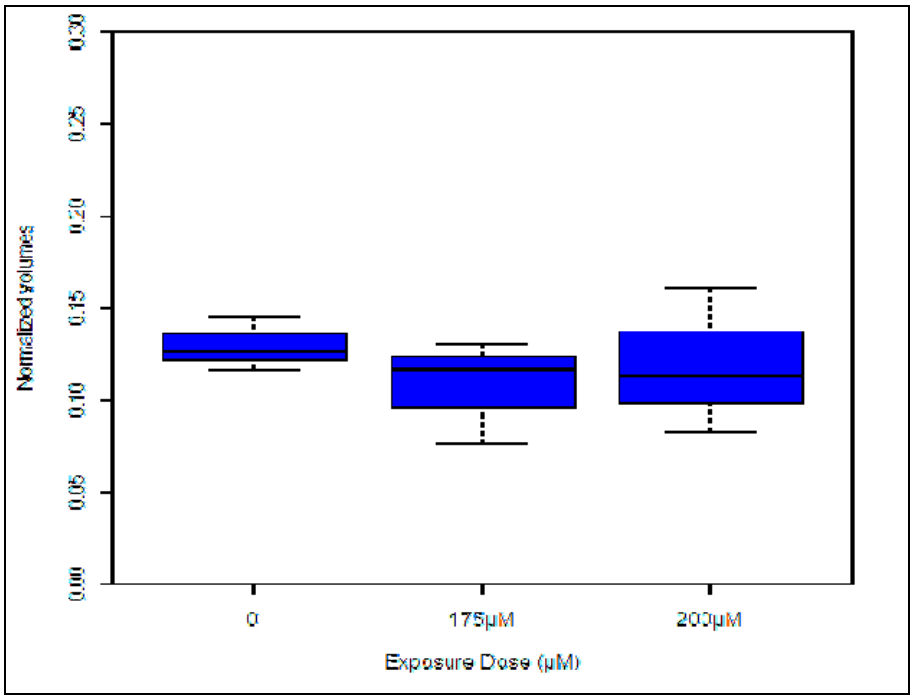


Figure 4.17: Average levels of BAX normalized with b-actin as loading control in H295R cells exposed to PFNA compared to DMSO 0.1 % (shown as 0 in the figure). The boxes in the box plot contain the middle 50 % of the data (25^{th}-75^{th} percentile). The line inside the boxes indicates the median, and the whiskers indicate the maximum and the minimum data value.

4.4.3 VIM

Exposure to PFNA gave a significant increase in vimentin expression compared to DMSO 0.1 % in the highest exposure dose (p=0.0031), and a borderline significant increase in 175 µM (p=0.0990) as shown in the figure below (Fig 4.20). PFOS exposure was not significant different from the corresponding concentrations of TBA (Fig 4.19). A picture of the membrane is shown in Fig 4.18.

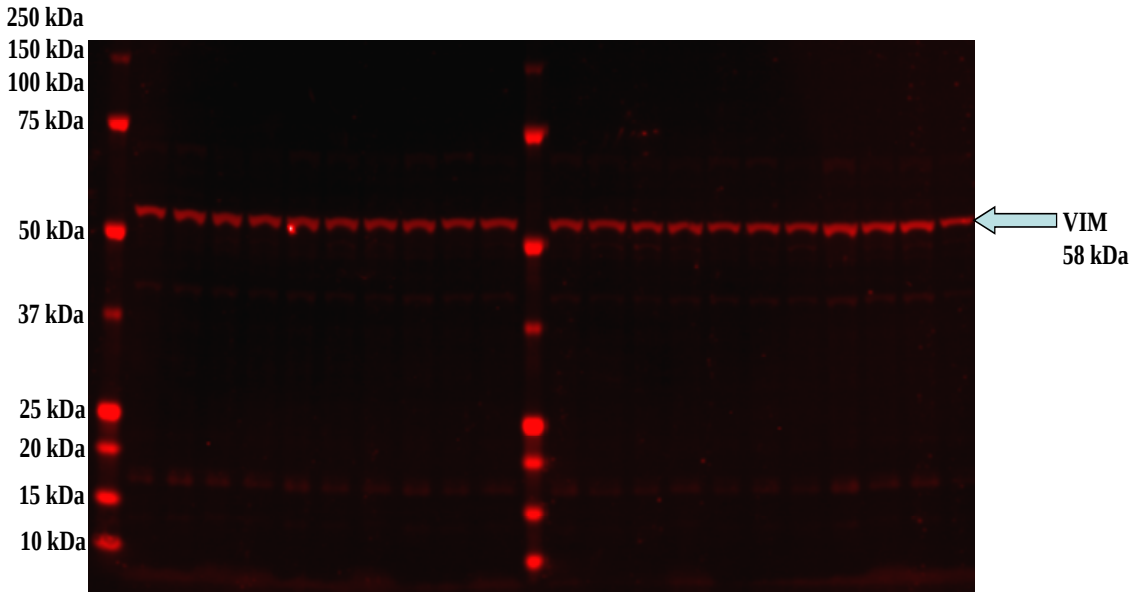


Figure 4.18: Proteins pools from H295R cells exposed to PFOS and PFNA were electrophorized and transferred to a membrane, with TBA and DMSO 0.1 % as their respective controls. The membrane was coated with antibody against VIM (MW 58 kDa), and thereby visualized, as seen in this figure. The protein pools from the cells were run as triplicates in a random order on the membrane. In addition a molecular weight marker was added in the first lane and in the middle lane, to determine the size of the antibody (MW listed to the left). The arrow at the right points at the bands used in statistics.

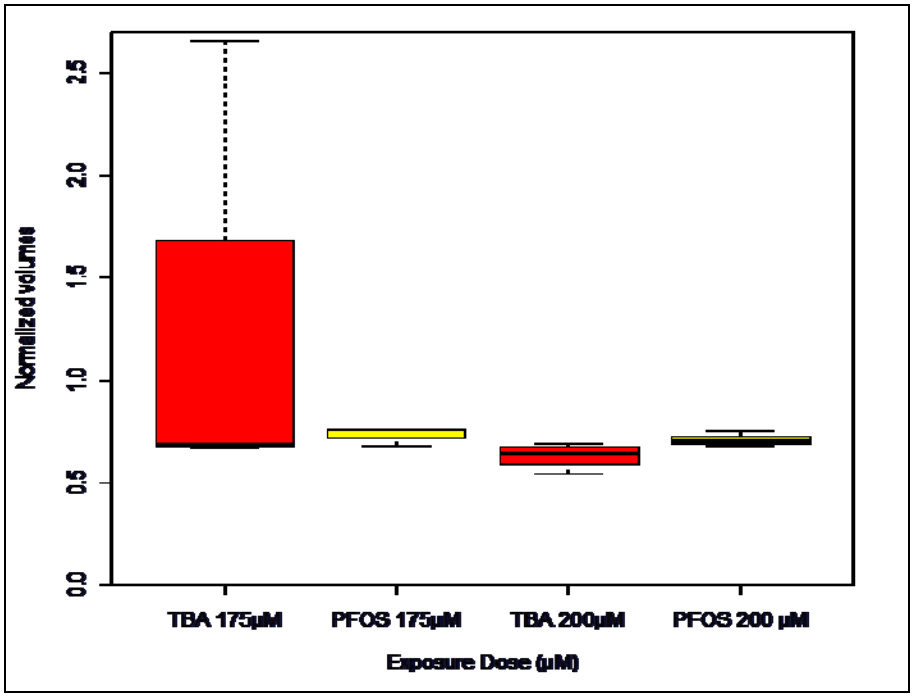


Figure 4.19: Average levels of VIM normalized with b-actin as loading control in H295R cells exposed to PFOS compared to corresponding concentrations of the control TBA. The boxes in the box plot contain the middle 50 % of the data (25^{th}-75^{th} percentile). The line inside the boxes indicates the median, and the whiskers indicate the maximum and the minimum data value. The red boxes represent cells exposed to TBA, and the yellow boxes represent cells exposed to PFOS.

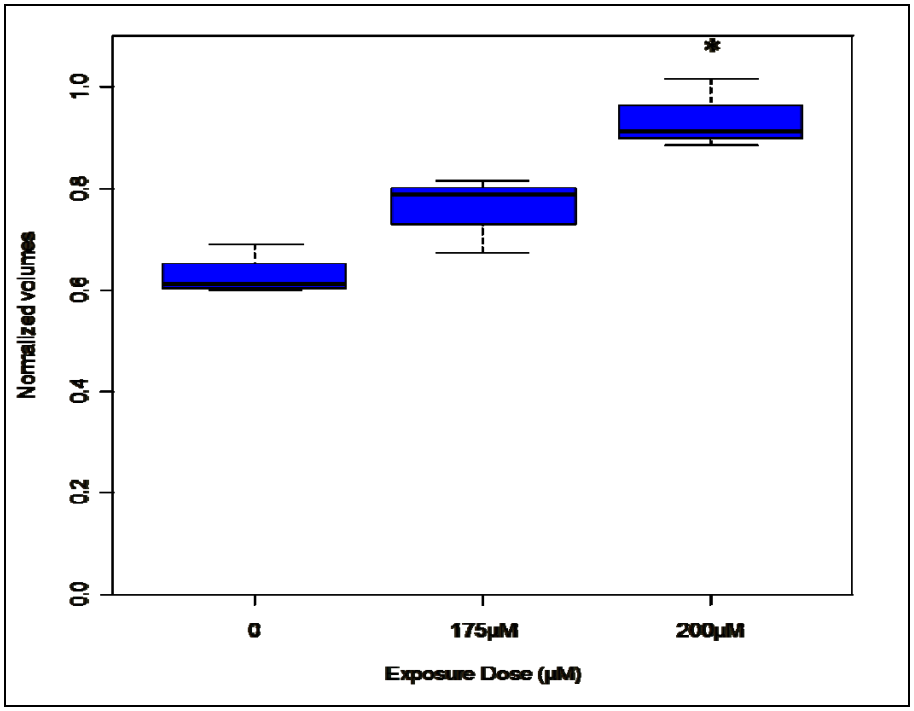


Figure 4.20: Average levels of VIM normalized with b-actin as loading control in H295R cells exposed to PFNA compared to DMSO 0.1 %. The boxes in the box plot contain the middle 50 % of the data (25^{th}-75^{th} percentile). The line inside the boxes indicates the median, and the whiskers indicate the maximum and the minimum data value. *: Significantly different from DMSO 0.1 % control within exposure group (P<0.05). The significance was tested by using Tukey-Kramer HSD Test in JMP8 Software.

4.4.4 StAR

None of the exposures showed any significant differences to their respective controls in the expression of StAR, as shown in the figures below (Figs 4.22 and 4.23). A picture of the membrane is shown in Fig 4.21.

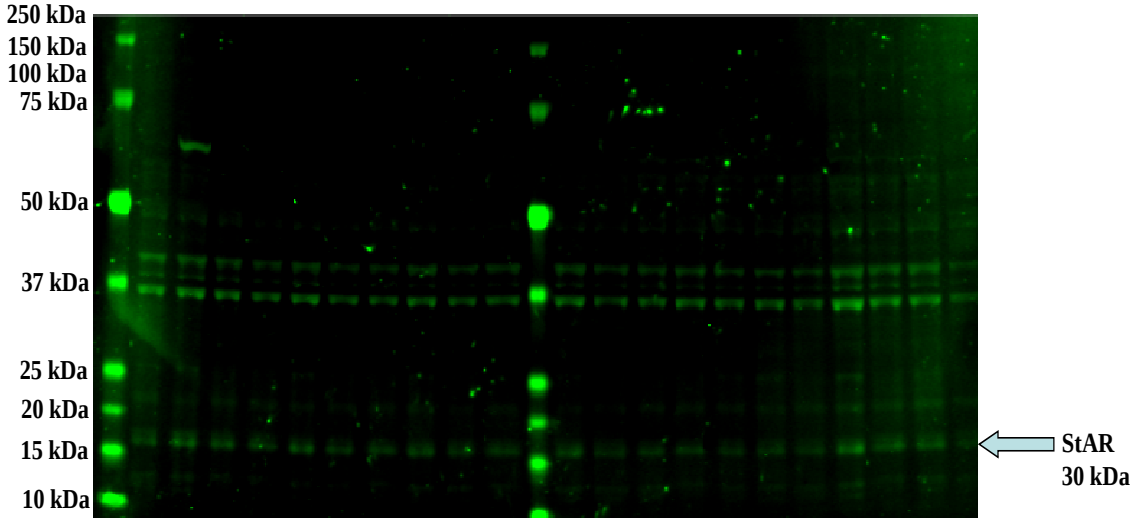


Figure 4.21: Proteins pools from H295R cells exposed to PFOS and PFNA were electrophorized and transferred to a membrane, with TBA and DMSO 0.1 % as their respective controls. The membrane was coated with antibody against StAR (MW 30 kDa), and thereby visualized, as seen in this figure. The protein pools from the cells were run as triplicates in a random order on the membrane. In addition a molecular weight marker was added in the first lane and in the middle lane, to determine the size of the antibody (MW listed to the left). The arrow at the right points at the bands used in statistics.

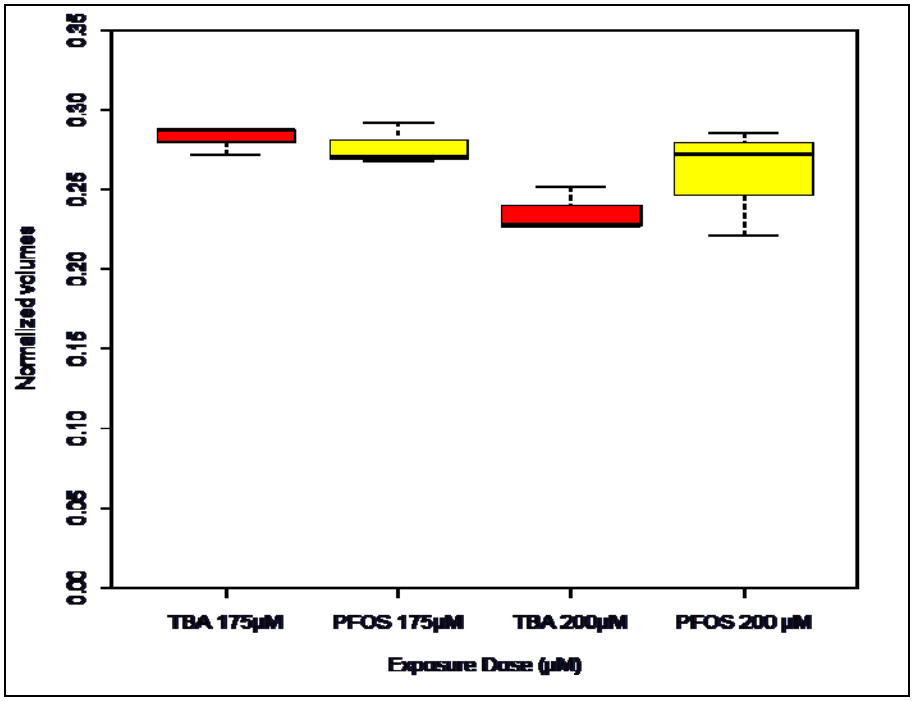


Figure 4.22: Average levels of VIM normalized with b-actin as loading control in H295R cells exposed to PFOS compared to corresponding concentrations of the control TBA. The boxes in the box plot contain the middle 50 % of the data (25th-75th percentile). The line inside the boxes indicates the median, and the whiskers indicate the maximum and the minimum data value. The red boxes represent cells exposed to TBA, while the yellow boxes represent cells exposed to PFOS.

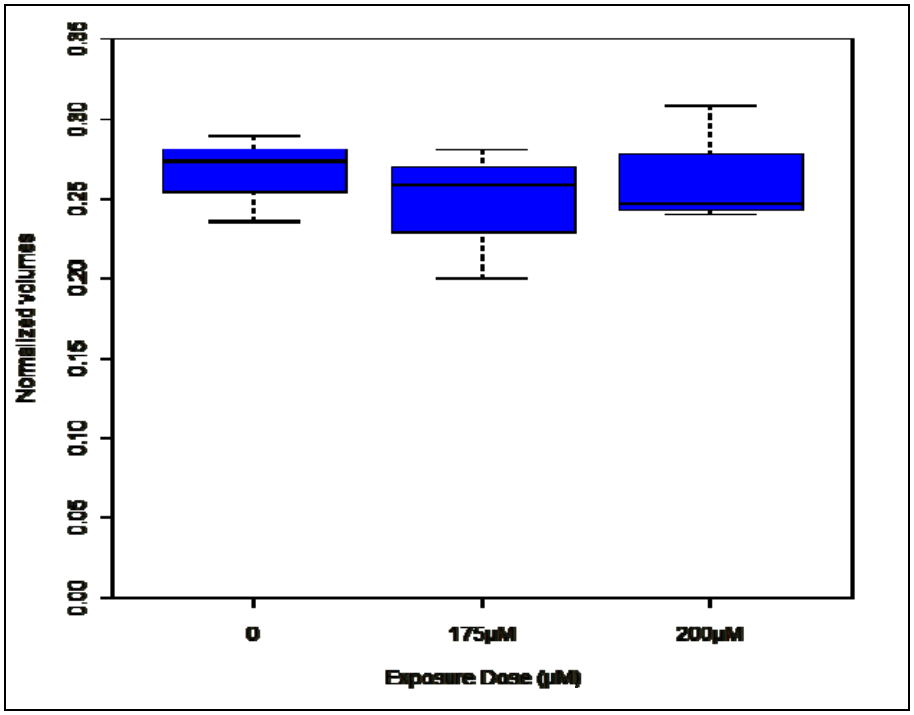


Figure 4.23: Average levels of StAR normalized with b-actin as loading control in H295R cells exposed to PFNA compared to DMSO 0.1 % (shown as 0 in the figure). The boxes in the box plot contain the middle 50 % of the data (25th-75th percentile). The line inside the boxes indicates the median, and the whiskers indicate the maximum and the minimum data value.

4.4.5 HSP70

Exposure to PFOS and PFNA did not give any significant differences in expression of HSP70, as shown in the figures below (Figs 4.25 and 4.26). However exposure to TBA 175 µM gave a statistically significant decrease in HSP70 expression compared to DMSO (p=0.0262) (Fig 4.25). A picture of the membrane is shown in Fig 4.24.

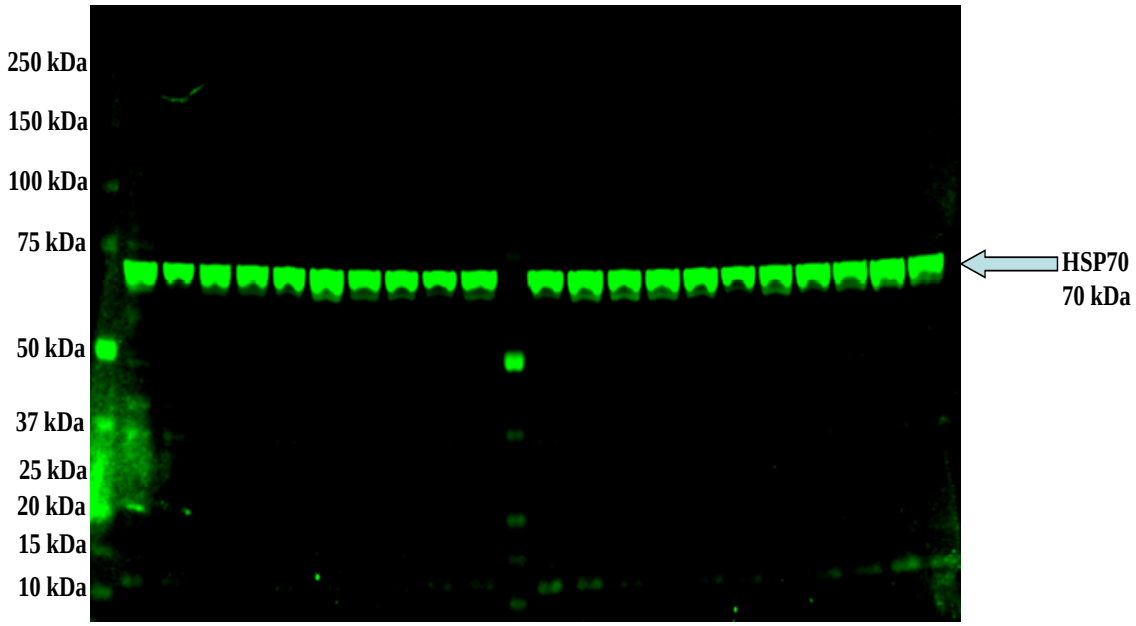


Figure 4.24: Proteins pools from H295R cells exposed to PFOS and PFNA were electrophorized and transferred to a membrane, with TBA and DMSO 0.1 % as their respective controls. The membrane was coated with antibody against HSP70 (MW 70 kDa), and thereby visualized, as seen in this figure. The protein pools from the cells were run as triplicates in a random order on the membrane. In addition a molecular weight marker was added in the first lane and in the middle lane, to determine the size of the antibody (MW listed to the left). The arrow at the right points at the bands used in statistics.

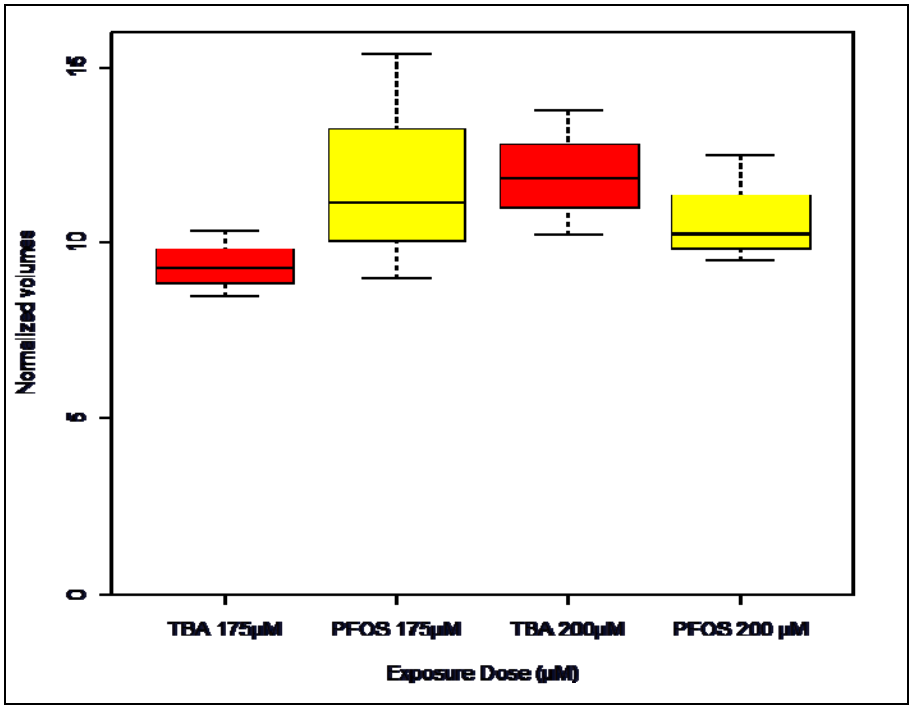


Figure 4.25: Average levels of HSP70 normalized with b-actin as loading control in H295R cells exposed to PFOS compared to corresponding concentrations of the control TBA. The boxes in the box plot contain the middle 50 % of the data (25^{th}-75^{th} percentile). The line inside the boxes indicates the median, and the whiskers indicate the maximum and the minimum data value. The red boxes represent cells exposed to TBA, while the yellow boxes represent cells exposed to PFOS.

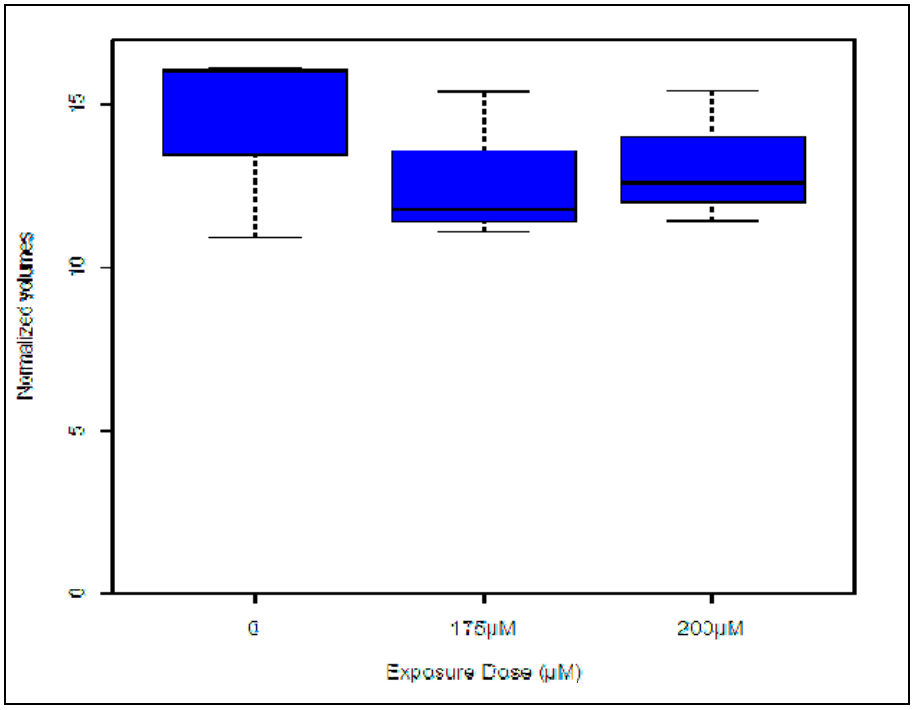


Figure 4.26: Average levels of HSP70 normalized with b-actin as loading control in H295R cells exposed to PFNA compared to DMSO 0.1 % (shown as 0 in the figure). The boxes in the box plot contain the middle 50 % of the data (25^{th}-75^{th} percentile). The line inside the boxes indicates the median, and the whiskers indicate the maximum and the minimum data value.

5. Discussion

To my knowledge, this is the first exposure study investigating both endocrine effects and alterations in protein expression in the H295R cell-line after exposure to PFOS and PFNA.

In the present study significant effects of PFOS and PFNA on adrenal steroidogenesis and protein expression were found. The general pattern of steroid response with the two compounds was a decrease in hormone secretion. PFOS proved to be cytotoxic, but the cytotoxicity was not dose dependent.

Allthough the mechanisms as to which PFCs alter cell functions in adrenal cells are not known, the current study provided new knowledge on how these compounds affected protein expression and might contribute to a better understanding of mechanisms related to PFC exposure.

The occurrence of PFCs in the environment has been reported to vary greatly, with highest levels around industrial areas. The highest levels of PFC has been found in mice inhabiting areas around a fluorotelomer plant in Belgium (180 000 ng/g). In invertebrates, fish and humans the highest levels reported are 2-280 ng/g, 3250 ng/g and 73 ng/ml respectively (Houde et al. 2006). The exposure doses used in this study were far higher compared to natural occurring levels of PFCs. However, due to accumulation of PFCs in protein-rich tissues and slow elimination rate of PFCs, there might be higher doses found naturally in target organs in organisms than reported.

5.1 Viability

The viability for H295R cells exposed to PFOS was decreased in all the three doses although there was no significant dose response relationship. Since all the doses gave significantly lower viability, the doses used for PFOS seem to have a cytotoxic effect and lower doses should have been tested. The decreased viability must be considered for the other results. However, significant dose-response relationships at hormone levels (this will be discussed later) indicated that other endpoint analyses were not influenced by the reduced viability.

The cells exposed to PFOS, were also exposed to TBA since the PFOS came as a tetrabutylammonium-salt. This may affect the cells, although there was no decrease in viability in cells exposed only to TBA dissolved in DMSO. TBA is a non-selective potassium channel blocker which may change the membrane potential (Sohn et al. 2000). In a study on human endothelial cells, the viability of cells exposed to TBA was not changed compared to controls (Krötz et al. 2002) as seen in this study. PFOS have been found to increase the permeability for other compounds in fish leukocytes while being inactive itself (Hu et al. 2003). Due to the previous findings there was reason to believe that the alterations in viability may partly be caused by the combination of PFOS and TBA.

Decreased cell viability in tilapia hepatocytes exposed to PFOS has been reported (Liu et al. 2007b). A possible explanation was suggested to be oxidative stress caused by PFOS and thereby production of ROS. For the current study, proteins found regulated as a response to PFCs indicate that oxidative stress occurs in the cells (this will be discussed later). A future perspective would be if oxidative damage might be an explanation for the decreased viability. Viability was not affected in hepatocytes from Atlantic salmon exposed to PFOS (Krøvel et al. 2008), and this illustrates that there is interspecies variation in the response to PFOS.

In the current study, the viability of H295R cells exposed to PFNA was not altered. In contrary, PFNA has been found to cause apoptosis in rat spleen in a previous study, where the apoptosis was suggested to be related to oxidative stress and mitochondria independent of caspase death signal pathway (Fang et al. 2010).

5.2 Endocrine effects – hormone quantification

All the measured hormones were altered after exposure to PFOS or PFNA. PFOS has been reported to stimulate the steroid production at lower exposure doses, and inhibit the production at higher exposure doses. The stimulatory effect was suggested to be caused either by stimulatory effects of ROS or inhibition in steroid clearance mechanisms (Oakes et al. 2005). The inhibition of steroid production might cause reduced cholesterol or reduced transport of cholesterol to the mitochondria for conversion to steroid hormones (Oakes et al. 2005).

5.2.1 Oestradiol

The increased production of oestradiol found in the cells exposed to PFOS revealed a tendency of a negative dose-response. Since the viability did not have the same negative dose-response relationship, the increase in oestradiol levels at the lowest exposure dose (150 μM) cannot be explained by a change in viability.

Several previous studies have shown increased oestradiol levels induced by perfluorinated compounds (Biegel et al. 1995; Liu et al. 2007a) as seen in this study.

An unpublished study (Kraugerud et. al 2010, manuscript in preparation), showed increased oestradiol levels in the same cell system as used in this study (H295R). However this study used other concentrations and another exposure design – the cells were exposed in 24 well plates as opposed to exposure in 75 cm^2 tissue culture flasks in this study. The concentrations of PFOS and PFNA in the previous study ranged from 6 nM to 600 μM. When recalculating the amount of compound per cell, the doses used in the current study were nearly equivalent to the next highest exposure dose (60 μM) in the previous study (see Table 3.1 in Material and Methods). However the increased levels of oestradiol at the lowest exposure dose in the current study (150 μM) do not correspond to the previous study in which the increase was found at the highest dose, not at the next highest dose. The fact that different results were obtained with the same cell line using different exposure design can indicate that this *in vitro* cell assay is sensitive to small changes. This might also be caused by different operators when conducting the study.

5.2.2 Testosterone

Cells exposed to PFOS and PFNA gave significant decreased secretion of testosterone. This correlates with previous *in vitro* studies done in Leydig cells exposed to PFOA (Biegel et al. 1995) and in testes from rats exposed to perfluorododecanoic acid (PFDoA) (Shi et al. 2007) where the levels of testosterone were significantly decreased.

Previous *in vivo* studies have also reported decreased levels of testosterone in rats exposed to PFNA (Feng et al. 2009) and decreased levels in testosterone in PFOA exposed rats corresponding to the observed decreases in testosterone in the current study (Biegel et al. 1995).

There was a trend in the data that testosterone decreased in a non-linear pattern with increasing concentrations of PFNA. This would agree with a previous report claiming that PFCs have nonlinear pharmacokinetics (Andersen et al. 2008)

5.2.3 Progesterone

Changes in progesterone levels as a consequence of exposure to PFOS or PFNA have to my knowledge not been reported in previous studies. However, a study done in testis from rats exposed to PFDoA, reported decreased serum progesterone levels (Shi et al. 2010). This corresponds to the results in the current study. In addition, a study done in rats, the corticosterone levels in PFOS exposed animals were increased (Austin et al. 2003). Another study reported decreased levels of corticosteroids in mice (Ribes et al. 2010). Several studies show decreased levels of cholesterol in different species, such as humans, monkeys, rats, mice and birds (Berthiaume et al. 2002; Hoff et al. 2005; Lin et al. 2009; Seacat et al. 2002). This indicates that the effects of PFOS and PFNA (due to similar modes of action) may affect mineralcorticoids both up-stream and down-stream of progesterone. However, the studies were done *in vivo* and the results cannot directly be compared to *in vitro* studies using a cell-line representing only one element of the HPA-axis, as in the current study.

5.2.4 Cortisol

Increase in cortisol levels due to exposure of PFCs have been reported in a study done in mice exposed to PFNA (Fang et al. 2008). In contrast to that report, the current study showed decreased cortisol levels. However, the previous study was done *in vivo* and reported increased ACTH levels in addition to the increased cortisol levels, and cannot be directly compared to the current study.

5.3 Protein profile

The H295R cells gave a clean profile on the 2D-gels. Few of the gels had smears, and although some contamination of keratin, most likely from making the gels or handling the gels, the spots were clear with defined peaks. This suggests that the H295R cell-line is

suitable for investigation of protein expression. There is only one previous study using H295R cells to investigate protein expression. The effects of mitotane on growth, steroidogenesis and proteomic profile were described, and the proteome profiling allowed identification of proteins related to different cellular function (Stigliano et al. 2008).

One study has investigated protein expression after exposure to PFCs. For zebra fish embryos exposed to PFOS, the proteomic profile included proteins involved in energy metabolism, cholesterol, oxidative stress, and membrane integrity (Shi et al. 2009). Compared to the current study, only one of the proteins was in the same family in the two studies; eukaryotic translation elongation factor 2 (current study) and eukaryotic translation elongation factor 1 (zebra fish study). These proteins will be further described later.

5.4 Identified proteins

Allthough the viability was decreased for cells exposed to PFOS, the same amount of proteins were added to the gels (800 µg). Thus the proteins identified have been regarded as truly regulated.

The proteomic profile from this study included proteins involved in several cellular processes which can be divided into different categories. Out of the 18 identified proteins found regulated, 3 proteins occurred in more than one spot (albumin, eEF2 and eIF4A1-Pdcd4 complex). That gave a total of 15 identified proteins found regulated. Out of the 15 identified proteins, at least 7 were found to be involved in transcription/protein synthesis, at least 3 were found to be involved in stress response, and at least 6 were found to be involved in carcinogenesis. There are limited informations about some of the proteins in response to exposure, and the mechanisms of action are not known. Thus discussing the regulated proteins was complicated and difficult.

5.4.1 Transport proteins

The increased levels of serum albumin found in the H295R cells exposed to PFOS and PFNA might be connected with PFCs affinity for binding to proteins. Albumin is a transport protein found in high concentrations in blood plasma, providing the cells with inorganic ions, amino acids, fatty acids etc. A previous study reported that PFOS

primarily binds to serum albumin in several organisms, thereby reducing the effects of the compound (Jones et al. 2003). Another report demonstrated an interaction between PFOA and serum albumin (Wu et al. 2009). Considering the chemical structure of PFCs, it is likely that PFNA also binds to albumin. It is unclear how the increased serum albumin in the current study can be explained, since it is not known that synthesis of this protein takes place in H295R cells. There was some serum albumin in the cell culture medium, but the increased albumin was measured from the cells, and not the medium. In addition, the increase was significant compared to the control. The strong binding of PFCs to serum albumin would logically result in decreased albumin levels in the cells, but instead elevated albumin levels were found in all the exposed cells except for one albumin isomere from cells exposed to PFNA. A reason for the increased serum albumin in cells exposed to PFOS might have been the capability of PFOS to increase the permeability for other compounds (Hu et al. 2003) and thereby caused a leakage of albumin from the cell culture medium into the cells.

5.4.2 Proteins involved in transcription/protein synthesis

In the current study high mobility group protein B1 (HMGB1) was decreased in cells exposed to both PFOS and PFNA. High mobility group proteins (HMG) are among the most ubiquitous, abundant and evolutionary conserved protein in eukaryotes. HMG proteins are divided into three different families; HMGA, HMGB and HMGN. HMGB1/2 are cytoplasmic proteins with the ability to migrate between the cytoplasm and nucleus dependent on cell-cycle. The roles of HMGB have been found to be involved both in DNA binding, promoting the transcription of several genes, and as cytokines and/or growth factors. HMGB1 establish protein-protein interactions with specific transcription factors, including all steroid nuclear receptors, enhances transcription from steroid receptor response elements by bending the DNA. HMGB1 is found to activate the NF-KB signaling pathway, and be involved in the recognition of distorted and damaged DNA structures, in maintenance of genome integrity, and in DNA repair (Bianchi et al. 2005; Czura et al. 2001). In addition HMGB1 has been found to increase the transcription when interacting with steroid hormone receptors (Thomas et al. 2001). Several studies have suggested that overexpression of HMGB is involved in development of cancer (Lange et al. 2009; Roesli et al. 2009). The decreased levels of HMGB1 in the exposed cells in the current study might partly be explained by the reports of increased release of HMGB1

into the extracellular medium by apoptotic and autophagic cells in response to stress or necrosis, to alert other cells about danger (Tang et al. 2010). Decreased viability in response to PFOS exposure might partly be explained by the down-regulation of HMGB1, since down-regulation of HMGB1 in prostate cancer cells has been reported to result in apoptosis (Tang et al. 2010). A future perspective would be to measure the levels of HMGB1 in the growth medium from the exposed cells.

The eIF4A-Pdcd4 complex was up-regulated in both cells exposed to PFOS and PFNA in the current study. Pdcd4 has been identified as a tumor suppressor shown to suppress neoplastic transformation and tumor phenotype. eIF4A functions as an ATP-dependent RNA helicase to catalyze unwinding of mRNA secondary structure. It mediates binding of mRNA to the 40S subunit of the ribosome (Quinn et al. 1999). The interaction between Pdcd4 and eIF4A inactivated the helicase function of eIF4A, suppresses cap-dependent translation and inhibits the activator protein 1 (AP-1) transactivation (Loh et al. 2009). The up-regulation of this complex suggests an increased inhibition of transcription through AP-1.

Eukaryotic initiation factor 4A2 (eIF4A2) were found to be increased in both cells exposed to PFOS and PFNA in the present study. eIF4A2 is one of three isoforms of eIF4A found in mammals (Li et al. 1999). It has shown to be down-regulated by high glucose levels in rat β-cells. This suggests that eIF4A2 may contribute to regulation of insulin production in response to glucose and thus may have a role in glucose homeostasis (Cheyssac et al. 2006). In addition, eIF4A2 has found to be a putative contributor to type 2 diabetes in presence of metabolic stress (Cheyssac et al. 2006). The up-regulation of this protein indicated an increased transcription.

In the present study, levels of eukaryotic initiation elongation factor 2 (eEF2) were found to be down-regulated in both cells exposed to PFOS and PFNA. eEF2 promotes the GTP-dependent translocation of the nascent protein chain from the A-site to the P-site of the ribosome (Ortiz et al. 2006). The down-regulation of eEF2 might be caused by phosphorylation and inactivation due cellular stress (Kang et al. 2007). In the current study eEF2 were found as two isomers. This suggests that the protein might have been post-translational altered (Kang et al. 2007).

The levels of eukaryotic initiation factor 3 subunit M (eIF3M) were increased in cells exposed to PFOS, and decreased in cells exposed to PFNA. Eukaryotic initiation factor 3

(eIF3) is a complex initiation factor that plays at least two roles in the protein synthesis; eIF3 binds to the 40S ribosome and facilitate binding of Met-tRNA/eIF2 complex to form the preinitiation complex, and secondly eIF3 assists eIF4 in recruiting mRNA to the ribosome (Zhou et al. 2005). Correlation between abnormal levels of eIF3 subunits and cancer suggest that regulation of eIF3 might be important in determining the balance between cell proliferation and apoptosis (Zhang et al. 2007). This correlated with the increased levels of eIF3M and the decreased viability in cells exposed to PFOS seen in this study.

In the current study, decreased levels of the protein upstream of N-ras (unr protein) were found in both cells exposed to PFOS and PFNA. The unr protein contains several cold shock domains (CSD) and are mainly found either in soluble form or associated to membranes, mainly ER, in the cytosol. They act as translational regulators and are involved in mRNA stability. The CSD of unr functions in binding proteins and nucleic acid, and show preference for unstructured single stranded DNA and RNA. It has been found to be essential due to a study where unr-deficient mice died at about 10 days of embryonic development (Mihailovich et al. 2010). The down-regulation of unr protein might be reflected in the reduced transcription factors found in the current study, as the unr proteins may act as translational regulators and are found to be involved in mRNA stability.

The actin-like 6A isoform 1 (ACTL6A) levels were increased in both cells exposed to PFOS and PFNA in the current study. ACTL6A belongs to a family of actin-related proteins and is associated with chromatin and involved in transcriptional regulation by establishment, remodeling and maintenance of chromatin structure (Harata et al. 1999). The increased protein expression of ACTL6A may suggest the increased need of chromatin remodeling as a response to exposure.

The down-regulation of some of the transcription factors may be the cells response to stress and repair mechanisms caused by the exposure. The transcription factors did not give information about which genes that may be down-regulated, only that there was a response in the cells. A reason for the down-regulation might have been the need of energy for other processes in the cell to deal with stress. RNA have been isolated from the cells, and as a further perspective, it would be interesting to see which genes are regulated after exposure of PFOS and PFNA.

Previous research has reported PFCs as peroxisome proliferating compounds as as agonists of PPAR which may cause a DNA binding-independent interference of transcription factors (Fang et al. 2008). This might be reflected in the decreased expression of transcription factors reported in the current study.

5.4.3 Structural proteins

In the current study, levels of vimentin were increased in both cells exposed to PFOS and PFNA. Vimentin is a structural protein in the class of intermediate filaments of the cell. The filaments are associated with both the plasma and the nuclear membrane (Ferrari et al. 1986). Lipid droplets in adrenal cells are attached to vimentin. Alterations in vimentin might affect cells by decreasing the efficiency of some transport processes. Vimentin might bind proteins that play a role in the intracellular transport of lipids (Gillard et al. 1998).

Vimentin is related to oxidative stress by alternation in cytoskeleton organization and induction of cytotoxicity (Hung et al. 2009), and is found to be a major molecule initiated and promoted by persistent oxidative stress (Tsubota et al. 2010). The increased vimentin in the current study might have been a response to oxidative stress.

5.4.4 Stress related proteins

Several studies have reported oxidative stress in response to exposure of PFCs (Fang et al. 2010; Liu et al. 2007b; Oakes et al. 2005). The heat shock protein HSP70 was increased in both cells exposed to PFOS and PFNA. Heat shock proteins are involved in the folding and assembly of proteins in ER, and might play a role in monitoring the transport of proteins through the cell. They have been found to have a protective role during cellular stress. Some of the functional properties are folding of proteins, maintenance of proteins in native folded states and repair of promotion of the degradation of misfolded proteins. In addition, the heat shock proteins modulate the progression and/or engagement of apoptosis (Huang et al. 2001). This might have had an effect on the viability for cells exposed to PFOS as the fold change was high for both concentrations (fold change =1.6 and 2.0).

Mice exposed to thermal stress resulted in rapid induction and expression of HSP70, especially in organs not constitutively expressing HSP70, including the liver, pancreas, heart, lung, adrenal cortex and intestine (Huang et al. 2001). Gene expression studies in Atlantic salmon hepatocytes exposed to PFOS have shown enhanced cellular stress. The mechanism is not known, but HSP70 gene expression was elevated (Krøvel et al. 2008) as in the present study.

HSP70 and vimentin is reported to interact and colocalize in response to stress (Evans 1998), and this correlates with increased levels of both HSP70 and vimentin in the current study.

The levels of protein disulfide isomerase A4 (PDIA4) were increased in both cells exposed to PFOS and PFNA in the current study. PDI is a family of proteins which functions as chaperones that catalyzes thiol sulfide exchange, mainly in the ER, to ensure the correct folding of nascent proteins (Stockwin et al. 2007). The proteins of the PDI family are found to bind specifically to DNA sequences, encoding DNA repair proteins. This suggests that they play a role in regulating stress response genes (Huang et al. 2009). A gene expression study done in hepatocytes from Atlantic salmon suggests that PDI plays an important protective role in cellular stress response to PFOS, in addition to its isomerase activities (Huang et al. 2009). This correlates with the increased fold changes (1.6 and 2.0) of PDIA4 seen after exposure of PFOS of the H295R cells in this study. The up-regulation of PDIA4 might be associated with increased ACTL6A due to the DNA repair capabilities of PDIA4 and ACTL6A capabilities of remodeling chromatin structure (Harata et al. 1999). In addition PDI has been reported to be one of the key elements in development of human lung cancer (Bührens et al. 2009).

5.4.5 Lipid synthesis

In the current study, triosephosphate isomerase (TPI) was increased both in cells exposed to PFOS and PFNA. TPI is an enzyme that catalyzes the conversion of dehydroxyacetone phosphate and glyceraldehyd 3-phosphate in the gluconeogenesis and glycolysis pathway. It is also important in the synthesis of fatty acids and the penthose-phosphate shunt, where glucose is oxidized coupled to the NADPH synthesis. The TPI gene is characterized as a housekeeping gene because the expression of the gene is found in all cell types, and is found to be required for cell growth and maintenance (Brown et al.

1985). Due to the involvement in the glucolytic pathway, the increase of TPI in the current study may be related to requirement of energy due to increased stress in the exposed cells (Di Michele et al. 2010). TPI has been reported to play a role in pathology of human cancer (Bührens et al. 2009; Di Michele et al. 2010; Roth et al. 2010).

5.4.6 Cell cycle progression

In the current study, the NEDD8 activating enzyme E1 was found to be up-regulated in both cells exposed to PFOS and PFNA. NEDD8 is an ubiquitin-like molecule, and has been found to be essential both mitotic and endoreduplicative cell cycle progression. It is essential for regulation of protein degradation pathways involved in cell cycle progression and morphogenesis (Tateishi et al. 2001).

The enzymes responsible for conjugating ubiquitin into substrates can be divided into three classes; ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin-protein ligases (E3) (Liakopoulos et al. 1998). In previous reports, it has been suggested that the ubiquitin-proteasome pathway may play an important role in regulation of nuclear receptors levels and restricting the duration and magnitude of receptor activity in response to hormones. Uba3 is the catalytic subunit of NEDD8 activating enzyme, and is found to significantly repress reporter gene expression mediated by steroid receptors. Uba3 does not interfere with the initiation of receptor-mediated transcription activity, but it is suggested to suppress the steroid receptor activity by promotion the termination of receptor-mediated gene transcription (Fan et al. 2002). This is interesting because of the decreased secreted levels of hormones measured in medium from the exposed cells in this study.

5.4.7 Energy production

There was an increase in ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit isoform a (ATP5A1) in cells exposed to PFOS and the middle dose of PFNA (175 μM), while the highest dose of PFNA caused a decreased level of the protein in the cells. ATP5A1 encodes a subunit of mitochondrial ATP synthase. ATP synthase catalyzes the synthesis of ATP to ADP. ATP5A1 is a putative modifier gene which regulates tumor development. Absence of ATP5A1 leads to apoptosis of the cell (Seth et

al. 2009). The increased need of energy due to stress might explaine the increased levels of ATP5A1 in the present study.

5.4.8 Purine salvage pathway

The levels of phosphoribosyl transferase domain containing 1 (Prtfdc1) were increased in both cells exposed to PFOS and PFNA. Prtfdc1 is a protein that is involved in the purine salvage pathway in which produces purine nucleosides from derivates without de novo synthesis. Disorder in the purine metabolism can lead to immunodeficiency (Nyhan 2005). The increase of Prtfdc1 in the current study might be due to increased DNA repair as a response to stress.

5.4.9 Protein expression compared to gene expression

Gene expression studies have reported various changes in different organisms; salmon hepatocytes exposed to PFOS gave changed expression in genes included in stress, apoptosis and lipid metabolism (Krøvel et al. 2008), livers from PFOS exposed rats showed altered metabolic status (Bjork et al. 2008), PFOS exposed skin cells from dolphins altered genes involved in cellular stress response, cell cycle progression and proliferation, and protein translation (Mollenhauer et al. 2009), and chickens exposed to PFOS and PFOA altered genes involved in transport of electrons and oxygen, metabolism of lipids and fatty acids and protein amino acid phosphorylation and proteolysis (Yeung et al. 2007). These gene expression studies correlate with the current study when comparing the genes and the proteins. The genes found in the previous studies and the proteins found in this study were not the same, but they have similar functions such as lipid metabolism, apoptosis, cellular stress response and transcription.

After proteomic analysis, none of the key-enzymes included in the steroidogenesis were found to be affected by exposure to PFOS or PFNA. In a gene expression study, HMGR was reported down-regulated in H295R cells exposed to PFNA (Kraugerud et. al 2010, manuscript in preparation). However, only 17 of the 180 significant spots in the current study were identified. There might still have been regulations in proteins involved in steroidogenesis. If the RNA had been measured either by q-PCR or microarrays, it is possible that some of the key-enzymes in the steroidogenesis would be found up- or

down-regulated. Another possible explanation might have been a delayed response. A further perspective is to expose the cells the same way as in this study, but delay the isolation of proteins. This may result in more representative proteins being regulated in the response to exposure.

5.5 IPA network

In order to increase the understanding of the results of the proteomic analysis, it is important to understand the interactions between proteins. One way of doing this is to use network programs. These programs make networks based on their known interactions. Ingenuity Pathway Analysis Software (IPA) is one program used in making networks. This software defines functional networks based on the gene names of the proteins, and does not consider the origin of the tissue. Still, the network made by IPA gives an illustration of the interactions between the proteins found in this study. Thus the use of this program will be discussed instead of considered as a result.

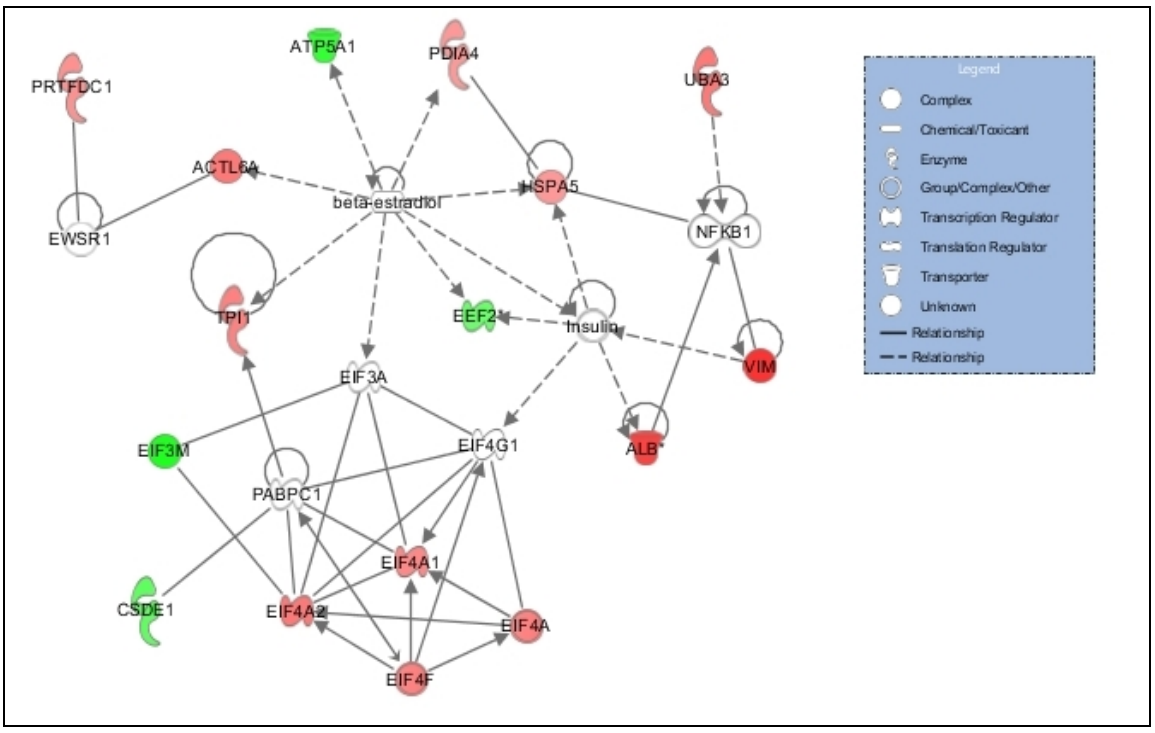


Figure 5.1: This figure is an illustration of the relation between the proteins found up- or down-regulated after proteomic analysis; 2D-gel and LC-MS/MS, of protein isolated from H295R cells exposed to PFOS and PFNA. This network was made by Ingenuity Pathway Analysis (IPA) by inserting the gene names of the detected proteins. The green symbols represent down-regulation, while the red symbols represent up-regulation. The white symbols represent proteins added by the software. Classifications of the proteins are explained in the blue box at the right.

Figure 5.1 shows a functional network identified by IPA. Proteins isolated from cells exposed to PFNA 200 μM were used to generate this network, but similar networks were identified for all exposures used, only with few differences in fold change. The relations between proteins are independent of up- or down-regulation of protein synthesis.

Interestingly nearly all of the proteins found regulated were connected in the same network (~95 %). Originally IPA made two separate networks, but due to similarities in functions, a merge of the networks was possible. The top part of the network, with beta-estradiol and insulin in central positions, was defined by IPA to be involved in cancer, cell-to-cell signaling and interaction, and tissue development. The lower part was defined to be involved in protein synthesis, gene expression and RNA trafficking.

The functions of the proteins found regulated in this study (colored red or green in figure) have been described above. For the genes found connected to the genes of the protein products in the lower network (labeled white), eIF3A is a translation initiation factor in the same family as eIF3M, and eIF4G1 is in the same family as eIF4A, eIF4A1, eIF4A2 and eIF4F, as described earlier. Poly (A) binding protein (PABP) is a cytosolic protein found to be ubiquitous and abundant. It is involved in mRNA translation by mediate mRNA circularization through binding of the eIF4F translation initiation complex which enhances translation by facilitating recycling of ribosomes. In addition PABP recruits various translation factors to the translating mRNA, and partners with the eukaryotic release factor eRE3 (Kozlov et al. 2001).

The top part of the network generated by IPA, representing genes involved in more general responses within the cell, have beta-estradiol in one of the central positions. This is interesting considering the H295R cells ability to perform full steroidogenesis. As mentioned above, oeastradiol measured in the medium collected from the exposed cells was increased, significantly in cells exposed to PFOS and borderline significant in cells exposed to PFNA. This may support the previous reported effects of PFOS related to oestrogen signaling. A similar trend, but not significant, was found for PFNA.

IPA gave insulin a central role in the top part of the network. Insulin has physiological effects on glucose, lipid and protein metabolism, and is shown to regulate cellular growth, differentiation and vascular function. Endocrine-disrupting chemicals may partly play a role in impairing the sensitivity of insulin. Non-esterified fatty acids, like PFCs, inhibit the carbohydrate metabolism and contribute to insulin resistance (Latini et al. 2009). A

recent study has shown a association between levels of PFCs in human serum and glucose homeostasis and indicators of metabolic disease (Lin et al. 2009). Endocrine disrupting chemicals have been reported to activate nuclear receptors and metabolic sensors, such as the PPARs. These have shown to be involved in the control of insulin sensitivity. PPARs are major regulators of lipid and glucose metabolism (Latini et al. 2009). Further, decreased levels of testosterone in human males have been associated with increased prevalence of obesity, insulin resistance and diabetes (Latini et al. 2009) which correspond to the findings in this study.

In addition to insulin and beta-estradiol, IPA connected two more genes to the top part of the network; EWSR1 and NF-KB. EWSR1 is found to be a transcription factor involved in ewing sarcoma (Ishida et al. 1998). NF-KB has found to be a transcription factor involved in regulating genes involved in inflammation and immune functions, and have been found to be stimulated by oxidative stress (Ginn-Pease et al. 1998).

The top part of the IPA network was defined to be involved in cancer, cell-to-cell signaling and interaction, and tissue development. This may indicate the carcinogenic effects of PFCs due to previous reports of the involvement of several of the regulated proteins in cancer development. Several studies have suggested that overexpression of HMGB is involved in development of cancer (Lange et al. 2009; Roesli et al. 2009). PDI has been reported to be one of the key elements in development of human lung cancer (Bührens et al. 2009) and TPI has been reported to play a role in pathology of human cancer (Bührens et al. 2009; Di Michele et al. 2010).

The lower part of the network was defined by IPA to be involved in transcription and protein synthesis. The effect on transcription after exposure to PFCs has already been addressed when discussing the regulated proteins.

5.6 Western blot

The proteins quantified in the western blot analysis were chosen based on knowledge about proteins involved in steroidogenesis and cellular stress. StAR and CYP17 were measured to see whether there was regulation of the steroidogenic enzymes. Neither CYP17 nor StAR showed any differences in regulation after exposure to PFOS or PFNA. This corresponded to the results of identified spots after the proteomic analysis.

Bcl-2-like protein4 (BAX) is an apoptosis regulator in the bcl-2 family. It accelerates programmed cell death by binding to and antagonizing the apoptosis repressor BCL2 (Alberts et al. 2004). BAX was quantified by western blot see whether the cells have an increased induction of apoptosis as a consequence of exposure. Cells exposed to PFOS had a decreased level of BAX. The decreased levels of BAX together with the reduced viability indicate that there is an increased level of apoptosis in these cells. Cell exposed to PFNA had not altered levels of BAX, which was a accordance with the viability test.

Vimentin and HSP70 were measured due to the findings after proteomic analysis. HSP70 was not regulated in the cells exposed to PFOS or the cells exposed to PFNA. This did not correspond to the regulation found after protein identification. A possible reason is that western blot measures the quantity of all the protein of interest, while identification done by proteomic analysis may have detected isomers. HSP70 may have been present in several spots in the 2D-gel, but only one of them was excised. Vimentin, however, was found up-regulated in the cells exposed to PFNA, but not regulated in cells exposed to PFOS. This correlated well with the LC-MS/MS. Vimentin was shown to be slightly up-regulated in cells exposed to PFOS (fold change 1.7 and 1.2), but not as much as in the cells exposed to PFNA (fold change 2.0 and 2.6), after protein identification.

5.7 Limitations to the current study

5.7.1 Viability

The decreased viability due to PFOS exposure was a great source of error in this study. The exposure doses used should have been lower to be able to have effects without the cytotoxicity, and to be able to detect NOAEL.

5.7.2 Number of replicates

In this study, 3 replicates were used for each exposure group. More replicates could have been conducted, but the experimental design caused limitations due to the huge amount of work and use of equipment.

5.7.3 TBA

As already mentioned, TBA is a non-selective potassium channel blocker which may change the membrane potential, without changing the viability (Sohn et al. 2000). However, expression of HSP70 measured by western blot, gave increased levels in H295R cells exposed to TBA. This could imply that TBA had a stress-like effect on the cells. In addition four of the identified spots were down-regulated compared to the solvent control, including albumin in two different isomers, HMG1 and TPI. Albumin, in both the isomers, was up-regulated in cells exposed to PFOS as a TBA-salt, while it was down-regulated in cells exposed to only TBA. HMG-1 is down-regulated in both cells exposed to PFOS as a TBA-salt, and TBA. TPI is down-regulated in cells exposed to TBA, while it is up-regulated in cells exposed to PFOS as a TBA-salt. These differences in up- and down-regulation suggest that TBA has an independent effect. A previous study indicated that PFOS may increase the permeability for other compounds (Hu et al. 2003), and it cannot be excluded that effects seen in cells exposed to PFOS could have been influenced by TBA.

5.7.4 Limitations to proteomics

Proteomic analysis gives a better understanding of disease processes and mechanisms of action than genomics. However, when interpreting results obtained in the present study, there were some limitations set by the method used. Firstly, the proteins are separated by both size and isoelectric pH and thus one protein could have been separated in several isomers. This means that one up-regulated or down-regulated spot did not necessarily represent the total amount of one protein, only regulation in one of the isomers. Secondly, one spot was not necessarily one protein, but might have consisted of several proteins. The software used in detecting the proteins uses a MOWSE score (Pappin et al. 1993), among other factors, to be able to say which of the proteins were most likely to be the most abundant protein in the spot. However smaller protein could have hidden in these spots. If a large spot was shown to be neither down- or up-regulated, there could still have been smaller proteins hiding which in fact were significantly regulated. Thirdly, the samples consisted of a large amount of proteins, while only a small number of proteins were identified due to limited resources. On the other hand, using Western blot, the total amount of the protein can be measured in a sample. Contrary to LC-MS, western blots

require knowledge on which proteins can be present in the sample based on the samples nature and the treatment.

Another limitation to proteomic analysis is the random selection of which protein spots to identify. Out of the 180 significant spots in this study, 17 were excised and identified. These 17 spots were subjectively elected based on significance, fold change and evaluation of the quality of the spots. Ideally, all the spots should have been identified to get a better understanding of the mechanisms following exposure to PFOS and PFNA. However, because of limited resources, both time and money, this was not possible to do.

6. Conclusions

Exposure to PFOS and PFNA caused both changes in protein expression and endocrine disrupting effects in the H295R *in vitro* cell model. The effects found in the H295R *in vitro* model, both the endocrine effects and alterations in proteins expression, cannot directly be transferred to *in vivo* systems. However, the alterations in hormones following exposure to PFCs have been reported earlier in both humans and wildlife. Hence, this study supports the PFCs effects on the hormone production and the endocrine effects of exposure to these compounds. The alterations in protein expression might also cause negative effects in *in vivo* mechanisms, but this should be futher studied.

The changes in protein regulation caused by PFOS and PFNA included several classes of proteins, such as transcription factors, transportation proteins and stress related proteins. This suggested that exposure to these compounds affects several cellular processes, including protein synthesis, stress response and apoptosis. In addition, many of the proteins found are reported to be involved in carcinogenesis.

The endocrine disrupting effects included changes in the hormone secretion; decreased levels of testosterone, progesterone and cortisol in both cells exposed to PFOS and PFNA. In addition oestradiol secretion increased in the cells. However, the increase was only statistically significant at the lowest concentration of PFOS.

After quantification of proteins by western blot, enzymes involved in the steroidogenesis were not altered. This might suggest that the observed changes in hormone concentrations occure down-stream of these enzymes. Elevated BAX levels confirmed the reduced viability in cells exposed to PFOS. The western blot analysis of vimentin confirmed results from the proteomic analysis. Western blot done for HSP70 did not correlate with the proteomic findings. Still this may have a reasonable explanation.

The H295R cell-line was found to be well suited for proteomic analysis, as the profile was clear with defined spots.

The detected negative effects of PFOS and PFNA, especially the potential carcinogenic effects, should be investigated further, and taken in consideration in future utilization of these compounds.

7. Future perspectives

All the interesting findings obtained from this study should be further investigated to be able to understand more details in mechanisms involved in exposure to PFCs.

It would be interesting to excise and identify more of the significant proteins to obtain more knowledge about PFCs' mode of action and interference in various cellular processes. In addition it would be interesting to expose the cells the same way as in this study (48h), but delay the isolation of proteins (24-48h). This may result in more representative proteins being regulated in the response to exposure.

Both RNA and DNA were isolated from the exposed cells, and it would be interesting to analyze the gene expression using qPCR or microarrays, and/or analyze the DNA for epigenetic changes or DNA damage.

The alterations of all the four hormones in the current study indicate the investigated compounds might affect hormonal axes in exposed individuals. It would be a future perspective to perform the same exposure in systems investigating the other parts of the HPA-axis, or in living animals with intact hormonal axes.

Previous studies have reported of oxidative stress caused by PFCs in which was confirmed in this study by alterations in stress related proteins. Further studies could be done in H295R cells to investigate the mechanisms behind oxidative stress caused by PFCs.

References

3M (2000). Phase Out Plan for PFOS-based Products, US. Environmental Protection Agency Public Docket AR226-0588. St. Paul, USA.

Adams, L. D. and Gallagher, S. R. (2005). "Two-Dimensional Gel Electrophoresis." Current Protocols in Immunology(Chapter 8): Unit 8.5.

Alberts, B., Bray, D., Hopkin, K., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P. (2004). Essential Cell Biology. New York, Garland Science, Taylor & Francis Group.

Alexander, B. H. and Olsen, G. W. (2007). "Bladder Cancer in Perfluorooctanesulfonyl Fluoride Manufacturing Workers." Ann Epidemiol **17**: 471-478.

Andersen, M. E., Butenhoff, J. L., Chang, S. C., Farrar, D. G., Kennedy, G. L., Lau, C., Olsen, G. W., Seed, J. and Wallace, K. B. (2008). "Perfluoroalkyl Acids and Related Chemistries - Toxicokinetics and Modes of Action." Toxicological Sciences **102**(1): 3-14.

Apelberg, B. J., Witter, F. R., Herbstman, J. B., Calafat, A. M., Halden, R. U., Needham, L. L. and Goldman, L. R. (2007). "Cord Serum Concentrations of Perfluorooctane Sulfonate (PFOS) and Perfluorooctanoate (PFOA) in Relation to Weight and Size at Birth." Environmental Health Perspectives **115**(11): 1670-1676.

Austin, M. E., Kasturi, B. S., Barber, M., Kannan, K., MohanKumar, P. S. and MohanKumar, S. M. J. (2003). "Neuroendocrine Effects of Perfluorooctane Sulfonate in Rats." Environmental Health Perspectives **111**(12): 1485-1489.

Barrier, M. and Mirkes, P. E. (2005). "Proteomics in Developmental Toxicology." Reproductive Toxicology **19**: 291-304.

Begley, T. H., White, K., Honigfort, P., Twaroski, M. L., Neches, R. and Walker, R. A. (2005). "Perfluorochemicals: Potential Sources of and Migration from Food Packaging." Food Additives and Contaminants **22**(10): 1023-1031.

Berthiaume, J. and Wallace, K. B. (2002). "Perfluorooctanoate, Perflourooctanesulfonate, and N-ethyl Perfluorooctanesulfonamido Ethanol; Peroxisome Proliferation and Mitochondrial Biogenesis." Toxicology Letters **129**(1-2): 23-32.

Bianchi, M. E. and Agrest, A. (2005). "HMG Proteins: Dynamic Players in Gene Regulation and Differentiation." Current Opinion in Genetics & Develpment **15**: 496-506.

Biegel, L. B., Liu, R. C. M., Hurtt, M. E. and Cook, J. C. (1995). "Effects of Ammounium Perfluorooctonate on Leydig Cell Function: *In Vitro, in Vivo and ex Vivo* Studies." Toxicology and Applied Pharmacology **134**: 18-25.

Bjork, J. A., Lau, C., Chang, S. C., Butenhoff, J. L. and Wallace, K. B. (2008). "Perfluorooctane Sulfonate-Induced Changes in Fetal Rat Liver Gene Expression." Toxicology.

Blackstock, W. P. and Weir, M. P. (1999). "Proteomics: Quantitative and Physical Mapping of Cellular Proteins." TIBTECH **17**: 121-127.

Bláha, L., Hilscherova, K., Mazurová, E., Hecker, M., Jones, P. D., Newsted, J. L., Bradley, P. W., Gracia, T., Duris, Z., Horká, I., Holoubek, I. and Giesy, J. P. (2006). "Alterations of Steroidogenesis in H295R Cells by Organic Sediment Contaminants and Relationships to Other Endocrine Disrupting Effects." Environment International **32**: 749-757.

Bossi, R., Riget, F. F., Dietz, R., Sonne, C., Fauser, P., Dam, M. and Vorkamp, K. (2005). "Preliminary Screening of Perfluorooctane Sulfonate (PFOS) and Other Fluorochemicals in Fish, Birds and Marine Mammals From Greenland and the Faroe Islands." Environmental Pollution **136**: 323-329.

Brown, J. R., Daar, I. O., Krug, J. R. and Maquat, L. E. (1985). "Characterization of the Functional Gene and Several Processed Psudogenes in the Human Triosephosphate Isomerase Gene Familiy." Molecular and Cellular Biology **5**(7): 1694-1706.

Burtis, C. A. and Ashwood, E. R. (2001). Tietz Fundamentals of Clinical Chemistry. Philadelphia, W.B. Saunders Company.

Bührens, R. I., Amelung, J. T., Reymond, M. A. and Beshay, M. (2009). "Protein Expression in Human Non-Small Cell Lung Cancer: A Systematic Database." Pathobiology **76**: 277-285.

Campbell, A. M. and Heyer, L. J. (2003). Discovering genomics, proteomics, and bioinformatics. San Fransisco, Pearson Education, Inc.

Cash, P., Argo, E. and Bruce, K. D. (1995). "Characterisation of Haemophilus Influenza Proteins by Two Dimensional Gel Electrophoresis." Electrophoresis **16**: 135-148.

Cash, P. and Kroll, J. S. (2003). "Protein Characterization by Two-Dimensional Gel Electrophoresis." Methods in Molecular Medicine **71**: 101-118.

Chambers, G., Lawrie, L., Cash, P. and Murray, G. I. (2000). "Proteomics: A New Approach to the Study of Disease." Journal of Pathology **192**: 280-288.

Champe, P. C. and Harvey, R. A. (1994). Biochemistry. Philadelphia, Lippincott Wiliams & Wilkins.

Cheyssac, C., Dina, C., Leprêtre, F., Vasseur-Delannoy, V., Dechaume, A., Lobbens, S., Balkau, B., Ruiz, J., Charpentier, G., Pattou, F., Joly, E., Prentki, M., Hansen, T., Pedersen, O., Vaxillaire, M. and Froguel, P. (2006). "EIF4A2 Is a Positional Canditate Gene at the 3q27 Locus Linked to Type 2 Diabetes in French Families." Diabetes **55**: 1171-1176.

Corsolini, S., Ademollo, N., Romeo, T., Greco, S. and Focardi, S. (2005). "Persistent Organic Pollutants in Edible Fish: A Human and Environmental Health Problem." Microchemical Journal **79**: 115-123.

Czura, C. J., Wang, H. and Tracey, K. J. (2001). "Dual Roles for HMGB1: DNA Binding and Cytokine." Journal of Endotoxin Research **7**(4): 315-321.

Dallaire, R., Ayotte, P., Pereg, D., Déry, S., Dumas, P., Langlois, E. and Dewailly, E. (2009). "Determinants of Plasma Concentrations of Perfluorooctanesulfonate and Brominated Organic Compounds in Nunavik Inuit Adults (Canada)." Environmental Health Perspectives **43**(13): 5130-5136.

Di Michele, M., Marcone, S., Cicchillitti, L., Corte, A. D., Ferlini, C., Scambia, G., Donati, M. B. and Rotilio, D. (2010). "Glycoproteomics of Paclitaxel Resistance in Human Epithelial Ovarian Cancer Cell Lines: Towards the Identification of Putative Biomarkers." Journal of Proteomics **73**: 879-898.

Ehrenstein, O. S. v., Fenton, S. E., Kato, K., Kuklenyik, Z., Calafat, A. M. and Hines, E. P. (2009). "Polyfluoroalkyl Chemicals in the Serum and Milk of Breastfeeding Women." Reproductive Toxicology **27**: 239-245.

Ehresman, D. J., Froehlich, J. W., Olsen, G. W., Chang, S. C. and Butenhoff, J. L. (2007). "Comparison of Human Whole Blood, Plasma and Serum Matrices for the Determination of Perfluorooctanesulfonate (PFOS), Perfluoroctanoate (PFOA), and Other Fluorochemicals." Environmental Research **103**: 176-184.

Ellis, D. A., Martin, J. W., De Silva, A. O., Mabury, S. A., Hurley, M. D., Andersen, M. P. S. and Wallington, T. J. (2004). "Degradation of Fluorotelomer Alcohols: A Likely Atmospheric Source of Perfluorinated Carboxylic Acids." Environmental Science & Technology **38**(12): 3316-3321.

ENV/JM/RD(2002)17/FINAL (2002). Organization for Economic Co-operation and Development.

Evans, R. B. (1998). "Vimentin: the Conundrum of the Intermediate Filament Gene Familiy." BioEssays **20**: 79-86.
Fan, M., Long, X., Bailey, J. A., Reed, C. A., Osborne, E., Gize, E. A., Kirk, E. A., Bigsby, R. M. and Nephew, K. P. (2002). "The Activating Enzyme of NEDD8 Inhibits Steroid Receptor Function " Molecular Endocrinology **16**(2): 315-330.
Fang, X., Feng, Y., Shi, Z. and Dai, J. (2009). "Alterations of Cytokines and MAPK Signalling Pathway are Related to the Immunotoxic Effect of Perfluorononanoic Acid " Toxicological Sciences **108**(2): 367-376.
Fang, X., Feng, Y., Wang, J. and Dai, J. (2010). "Perfluorononanoic Acid-Induced Apoptosis in Rat Spleen Involves Oxidative Stress and the Activation of Caspase-Independent Death Pathway." Toxicology **267**: 54-59.
Fang, X., Zhang, L., Feng, Y., Zhao, Y. and Dai, J. (2008). "Immunotoxic Effects of Perfluorononanoic Acid on BALB/c Mice." Toxicological Sciences **105**(2): 312-321.
Feng, Y., Shi, Z., Fang, X., Xu, M. and Dai, J. (2009). "Perfluorononanoic Acids Induces Apoptosis Involving the Fas Death Receptor Signaling Pathway in Rat Testis." Toxicology Letters **190**: 224-230.
Ferrari, S., Battini, R., Kaczmarek, L., Rittling, S., Calabretta, B., De Riel, K., Philiponis, V., Wei, J. F. and Baserga, R. (1986). "Coding Sequence and Growth Regulation of the Human Vimentin Gene." Molecular and Cellular Biology **6**(11): 3614-3620.
Fisher, B. E. (1999). "Most Unwanted Persistent Organic Pollutants." Environmental Health Perspectives **107**(1): A18-A23.
Fowler, P. A., Dorà, N. J., McFerran, H., Amezaga, M. R., Miller, D. W., Lea, R. G., Cash, P., McNeilly, A. S., Evans, N. P., Cotinot, C., Sharpe, R. M. and Rhind, S. M. (2008). "*In utero* Exposure to Low Doses of Environmental Pollutants Disrupts Fetal Ovarian Development in Sheep." Molecular Human Reproduction **14**(5): 269-280.
Fromme, H., Tittlemier, S. A., Völkel, W., Wilhelm, M. and Twardella, D. (2009). "Perfluorinated Compounds - Exposure Assessment for the General Population in Western Countries." International Journal of Hygiene and Environmental Health **212**: 239-270.
Gazdar, A. F., Oie, H. K., Shackleton, C. H., Chen, T. R., Triche, T. J., Myers, C. E., Chrousos, G. P., Brennan, M. F., Stein, C. A. and Larocca, R. V. (1990). "Establishment and Characterization of A Human Adrenocortical Carcinoma Cell-Line That Expresses Multiple Pathways of Steroid-Biosynthesis." Cancer Research **50**(17): 5488-5496.
Giesy, J. P. and Kannan, K. (2001). "Global Distribution of Perfluorooctane Sulfonate in Wildlife." Environmental Science & Technology **35**(7): 1339-1342.
Giesy, J. P. and Kannan, K. (2002). "Perfluorochemical Surfactants in the Environment." Environmental Science & Technology **36**: 147A-152A.
Gillard, B. K., Clement, R., Colucci-Guyon, E., Babinet, C., Schwarzmann, G., Taki, T., Kasama, T. and Marcus, D. M. (1998). "Decreased Synthesis of Glycosphingolipids in Cells Lacking Vimentin Intermediate Filaments." Experimental Cell Research **242**: 561-572.
Ginn-Pease, M. E. and Whisler, R. L. (1998). "Redox Signals and NF-KB Activation in T-Cells." Free Radical Biology & Medicine **25**(3): 346-361.
Goksøyr, A. (2006). "Endocrine Disruptors in the Marine Environment: Mechanisms of Toxicity and Their Influence on Reproductive Processes in Fish." Journal of Toxicology and Environmental Health, Part A **69**: 175-184.

Gracia, T., Hilscherova, K., Jones, P. D., Newsted, J. L., Higley, E. B., Zhang, X., Hecker, M., Murphy, M. B., Yu, R. M. K., Lam, P. K. S., Wu, R. S. S. and Giesy, J. P. (2007). "Modulation of Steroidogenic Gene Expression and Hormone Production of H295R Cells by Pharmaceuticals and Other Environmentally Active Compounds." Toxicology and Applied Pharmacology **225**(2): 142-153.

Gracia, T., Hilscherova, K., Jones, P. D., Newsted, J. L., Zhang, X. W., Hecker, M., Higley, E. B., Sanderson, J. T., Yu, R. M. K., Wu, R. S. S. and Giesy, J. P. (2006). "The H295R System for Evaluation of Endocrine-Disrupting Effects." Ecotoxicology and Environmental Safety **65**(3): 293-305.

Harada, K., Inoue, K., Morikawa, A., Yoshinaga, T., Saito, N. and Koizumi, A. (2005). "Renal Clearance of Perfluorooctane Sulfonate and Perfluorooctanoat in Humans and Their Species-Specific Excretion." Environmental Research **99**: 253-261.

Harata, M., Mochizuki, R. and Mizuno, S. (1999). "Two Isoforms of a Human Actin-Related Protein Show Nuclear Localization and Mutually Selective Expression between Brain and Other Tissues." Bioscience, Biotechnology and Biochemistry **63**(5): 917-923.

Harvey, P. W., Everett, D. J. and Springall, C. J. (2007). "Adrenal Toxicology: A Strategy for Assessment of Functional Toxicity to the Adrenal Cortex and Steroidogenesis." Journal of Applied Toxicology **27**: 103-115.

Haug, L. S., Thomsen, C. and Becher, G. (2009). "Time Trends and the Influence of Age and Gender on Serum Concentrations of Perfluorinated Compounds in Archived Human Samples." Environmental Science & Technology **43**(6): 2131-2136.

Haukås, M., Berger, U., Hop, H., Gulliksen, B. and Gabrielsen, G. W. (2007). "Bioaccumulation of Per- and Polyfluorinated Alkyl Substances (PFAS) in Selected Species From the Barents Sea Food Web." Environmental Pollution **148**: 360-371.

Hecker, M. and Giesy, J. P. (2008). "Novel trends in endocrine disuptor testing: the H295R Steroidogenesis Assay for identification of inducers and inhibitors of hormone production." Analytical and Bioanalytical Chemistry **390**: 287-291.

Hecker, M., Newsted, J. L., Murphy, M. B., Higley, E. B., Jones, P. D., Wu, R. and Giesy, J. P. (2006). "Human Adrenocarcinoma (H295R) Cells for Rapid *in vitro* Determination of Effects on Steroidogenesis: Hormone Production." Toxicology and Applied Pharmacology **217**: 114-124.

Heijne, W. H. M., Kienhuis, A. S., van Ommen, B., Stierum, R. H. and Groten, J. P. (2005). "System Toxicology: Applications of Toxigenomics, Transcriptomics, Proteomics and Metabolomics in Toxocology." Expert Reviews of Proteomics **2**(5): 767-780.

Hilscherova, K., Jones, P. D., Gracia, T., Newsted, J. L., Zhang, X., Sanderson, J. T., Yu, R. M. K., Wu, R. S. S. and Giesy, J. P. (2004). "Assessment of the Effects of Chemicals on the Expression of Ten Steroidogenic Genes in the H295R Cell Line Using Real-Time PCR." Toxicological Sciences **81**: 78-89.

Hoff, P. T., Van de Vijver, K., Dauwe, T., Covaci, A., Maervoet, J., Eens, M., Blust, R. and De Coen, W. (2005). "Evaluation of Biochemical Effects Related to Perfluorooctane Sulfonic Acid Exposure in Organohalogen-contaminated Great Tit (Parus major) and Blue Tit (Parus caeruleus) Nestlings." Chemosphere **61**(11): 1558-1569.

Houde, M., Martin, J. W., Letcher, R. J., Solomon, K. R. and Muir, D. C. G. (2006). "Biological Monitoring of Polyfluoroalkyl Substances: A Review." Environmental Science & Technology **40**(11): 3463-3473.

Hu, W., Jones, P. D., Celius, T. and Giesy, J. P. (2005). "Identification of Genes Responsive to PFOS Using Gene Expression Profiling." Environmental Toxicology and Pharmacology **19**: 57-70.

Hu, W. Y., Jones, P. D., DeCoen, W., King, L., Fraker, P., Newsted, J. L. and Giesy, J. P. (2003). "Alterations in Cell Membrane Properties Caused by Perfluorinated Compunds." Comparative Biochemistry and Physiology, Part C **135**: 77-88.

Huang, L., Mivechi, N. F. and Moskophidis, D. (2001). "Insights into Regulation and Function of the Major Stress-Induced hsp70 Molecular Chaperone In Vivo: Analysis of Mice with Targeted Gene Disruption of the *hsp70.1* or *hsp70.3* Gene." Molecular and Cellular Biology **21**(24): 8575-8591.

Huang, T. S., Olsvik, P. A., Krøvel, A. V., Tung, H. S. and Torstensen, B. (2009). "Stress-Induced Expression of Protein Disulfide Isomerase Associated 3 (PDIA3) in Atlantic Salmon (*Salmo salar L.*)." Comparative Biochemistry and Physiology, Part B **154**: 435-442.

Hung, Y. C., Wang, P. W., Pan, T. L., Bazylak, G. and Leu, Y. L. (2009). "Proteomic Screening of Antioxidant Effects Exhibited by *Radix Salvia miltiorrhiza* Aqueous Extract in Cultured Rat Aortic Smooth Muscle Cells under Homocysteine Treatment." Journal of Ethnopharmacology **124**: 463-474.

Inoue, K., Okada, F., Ito, R., Kato, S., Sasaki, S., Nakajima, S., Uno, A., Saijo, Y., Sata, F., Yoshimura, Y., Kishi, R. and Nakazawa, H. (2004). "Perfluorooctane Sulfonate (PFOS) and Related Perfluorinated Compounds in Human Maternal and Cord Blood Samples: Assessment of PFOS Exposure in a Susceptible Population during Pregnancy." Environmental Health Perspectives **112**(11): 1204-1207.

Invitrogen (2003). Novex Pre-Cast Gel Electrophoresis Guide.

Ishibashi, H., Yamauchi, R., Matsuoka, M., Kim, J. W., Hirano, M., Yamaguchi, A., Tominaga, N. and Arizono, K. (2008). "Fluorotelomer Alcohols Induce Hepatic Vitellogenin Through Activation of the Estrogen Receptor in Male Medaka (*Oryzias latipes*)." Chemosphere **71**: 1853-1859.

Ishida, S., Yoshida, K., Kaneko, Y., Tanaka, Y., Sasaki, Y., Urano, F., Umezawa, A., Hata, J. and Fujinaga, K. (1998). "The Genomic Breakpoint and ChimericTranscripts in the EWSR1-ETV4/E1AF Gene Fusion in Ewing Sarcoma." Cytogenetics and Cell Genetics **82**: 278-283.

Jensen, A. A. and Leffers, H. (2008). "Emerging Endocrine Disrupters: Perfluoroalkylated Substances." International Journal of Andrology **31**: 161-169.

Johansson, N., Eriksson, P. and Viberg, H. (2009). "Neonatal Exposure to PFOS and PFOA in Mice Results in Changes in Proteins which are Important for Neuronal Growth and Synaptogenesis in the Developing Brain." Toxicological Sciences **108**(2): 412-418.

Jones, P. D., Hu, W., DeCoen, W., Newsted, J. L. and Giesy, J. P. (2003). "Binding of Perfluorinated Fatty Acids to Serum Proteins." Environmental Toxicological Chemistry **22**(11): 2639-49.

Kang, S., Kim, E. Y., Bahn, Y. J., Chung, J. W., Lee, D. H., Park, S. G., Yoon, T. S., Park, B. C. and Bae, K. H. (2007). "A Proteomic Analysis of the Effect of MAPK Pathway Activation on L-Glutamate-Induced Neuronal Cell Death." Cellular & Molecular Biology Letters **12**: 139-147.

Kannan, K., Corsolini, S., Falandysz, J., Fillmann, G., Kumar, K. S., Loganathan, B. G., Mohd, M. A., Olivero, J., Wouwe, N. V., Yang, J. H. and Aldous, K. M. (2004). "Perfluorooctanesulfonate and Related Fluorochemicals in Human Blood from Several Countries." Environmental Science & Technology **38**(17): 4489-4495.

Kannan, K., Corsolini, S., Falandysz, J., Oehme, G., Focardi, S. and Giesy, J. P. (2002a). "Perfluorooctanesulfonate and Related Fluorinated Hydrocarbons in Marine Mammals, Fishes, and Birds from Coasts of the Baltic and the Mediterranean Sea." Environmental Science & Technology **36**(15): 3210-3216.

Kannan, K., Koistinen, J., Beckmen, K., Evans, T., Gorzelany, J. F., Hansen, K. J., Jones, P. D., Helle, E., Nyman, M. and Giesy, J. P. (2001). "Accumulation of Perfluorooctane Sulfonate in Marine Mammals." Environmental Science & Technology **35**(8): 1593-1598.

Kannan, K., Newsted, J. L., Halbrook, R. S. and Giesy, J. P. (2002b). "Perfluorooctanesulfonate and Related Fluorinated Hydrocarbons in Mink and River Otters from the United States." Environmental Science & Technology **36**(12): 2566-2571.

Kannan, K., Tao, L., Sinclair, E., Pastva, S. D., Jude, D. J. and Giesy, J. P. (2005). "Perfluorinated Compounds in Aquatic Organisms at Various Trophic Levels in a Great Lakes Food Chain " Archives of Environmental Contamination and Toxicology **48**: 559-566.

Kavlock, R. J. and Ankey, G. T. (1996). "A Perspective on the Risk Assessment Process for Endocrine-Disruptive Effects on Wildlife and Human Health." Risk Analysis **16**(6): 731-739.

Kelly, B. C., Ikonomou, M. G., Blair, J. D., Surridge, B., Hoover, D., Grace, R. and Gobas, F. A. P. C. (2009). "Perfluoroalkyl Contaminants in the Arctic Marine Food Web - Trophic Magnification and Wildlife Exposure." Environmental Science & Technology **43**(11): 4037-4043.

Kleszczynski, K., Gardzielewski, P., Mulkiewicz, E., Stepnowski, P. and Skladanowski, A. C. (2007). "Analysis of Structure-cytotoxicity *in vitro* Relationship (SAR) for Perfluorinated Carboxylic Acids." Toxicology in Vitro **21**: 1206-1211.

KLIF (2008). TA-2395/2008: SFTs Arbeid med Perfluorerte Forbindelser 2008-2009. Oslo.

Kozlov, G., Trempe, J. F., Khaleghpour, K., Kahvejian, A., Ekiel, I. and Gehring, K. (2001). "Structure and Function of the C-Terminal PABC Domain of Human Poly(A)-Binding Protein." Proceedings of the National Academy of Science **98**(8): 4409-4413.

Krötz, F., Sohn, H. Y., Keller, M., Gloe, T., Bolz, S. S., Becker, B. F. and Pohl, U. (2002). "Depolarization of Endothelial Cells Enhances Platelet Aggregation Through Oxidative Inactivation of Endothelial NTPDase." Arteriosclerosis, Thrombosis, and Vascular Biology **22**: 2003-2009.

Krøvel, A. V., Søfteland, L., Torstensen, B. and Olsvik, P. A. (2008). "Transcriptional Effects of PFOS in Isolated Hepatocytes from Atlantic Salmon *Salmo Salar* L." Comparative Biochemistry and Physiology, Part C **148**: 14-22.

Kudo, N., Suzuki-Nakajima, E., Mitsumoto, A. and Kawashima, Y. (2006). "Responses of the Liver to Perfluorinated Fatty Acids with Different Carbon Chain Length in Male and Female Mice: In Relation to Induction of Hepatomegaly, Peroxisomal b-Oxidation and Microsomal 1-Acylglycerophosphocholine Acetyltransferase." Biological & Pharmaceutical Bulletin **29**(9): 1952-1957.

Kudo, N., Suzuki, E., Katakura, M., Ohmori, K., Noshiro, R. and Kawashima, Y. (2001). "Comparison of the Elimination Etween Perfluorinated Fatty Acids with Different Carbon Chain Length in Rats." Chemico-Biological Interactions **134**: 203-216.

Kärrman, A., Domingo, J. L., Llebaria, X., Nadal, M., Bigas, E., van Bavel, B. and Lindström, G. (2010). "Biomonitoring Perfluorinated Compounds in Catalonia,

Spain: Concentrations and Trends in Human Liver and Milk Samples." Environmental Science and Pollution Research **17**: 750-758.
Kärrman, A., Harada, K. H., Inoue, K., Takasuga, T., Ohi, E. and Koizumi, A. (2009). "Relationship Between Dietary Exposure and Serum Perfluorochemical (PFC) Levels - A Case Study." Environment International **35**: 712-717.
Lange, S. S. and Vasques, K. M. (2009). "HMGB1: The Jack-of-All-Trades Protein Is a Master DNA Repair Mechanic." Molecular Carcinogenesis **48**: 571-580.
Latini, G., Marcovecchio, M. L., Del Vecchio, A., Gallo, F., Bertino, E. and Chiarelli, F. (2009). "Influence of Environment on Insulin Sensitivity." Environment International **35**: 987-993.
Lau, C., Anitole, K., Hodes, C., Lai, D., Pfahles-Hutchens, A. and Seed, J. (2007). "Perfluoroalkyl Acids: A Review of Monitoring and Toxicological Findings." Toxicological Sciences **99**(2): 366-394.
Lau, C., Butenhoff, J. L. and Rogers, J. M. (2004). "The Developmental Toxicity of Perfluoroalkyl Acids and Their Derivates." Toxicology and Applied Pharmacology **198**: 231-241.
Lewin, B. (2008). Genes IX. Sudbury, Jones and Bartlett Publishers, Inc.
Li, L., Xu, Z. S. and Song, G. W. (2009). "Study on the Langmuir Aggregation of Fluorinated Surfactants on Protein " Journal of Fluorine Chemistry **130**: 225-230.
Li, Q., Imataka, H., Morino, S., Rogers JR, G. W., Richter-Cook, N. J., Merrick, W. C. and Sonenberg, N. (1999). "Eukaryotic Translation Initiation Factor 4AIII (eIF4AIII) Is Functionally Distinct from eIF4AI and eIF4AII." Molecular and Cellular Biology **19**(11): 7336-7346.
Li, Q. Q., Loganath, A., Chong, Y. S., Tan, J. and Obbard, J. P. (2006). "Persistent Organic Pollutants and Adverse Health Effects in Humans." Journal of Toxicology and Environmental Health, Part A **69**: 1987-2005.
Liakopoulos, D., Doenges, G., Matuschewski, K. and Jentsch, S. (1998). "A Novel Protein Modification Pathway Related to the Ubiquitin System." The EMBO Journal **17**(8): 2208-2214.
Lin, C. Y., Chen, P. C., Lin, Y. C. and Lin, L. Y. (2009). "Assiciation Among Serum Perfluoroalkyl Chemicals, Glucose Homeostasis, and Metabolic Syndrome in Adolecents and Adults." Diabetes Care **32**(4): 702-707.
Liu, C., Du, Y. and Zhou, B. (2007a). "Evaluation of Estrogenic Activities and Mechanisms of Action of Perfluorinated Chemicals Determined by Vitellogenin Induction in Primary Cultured Tilapia Hepatocytes." Aquatic Toxicology **85**: 267-277.
Liu, C., Yu, K., Shi, X., Wang, J., Lam, P. K. S., Wu, R. S. S. and Zhou, B. (2007b). "Induction of Oxidative Stress and Apoptosis by PFOS and PFOA in Primary Cultured Hepatocytes of Freshwater Tilapia (*Oreochromis niloticus*)." Aquatic Toxicology **82**: 135-143.
Loh, P. G., Yang, H. S., Walsh, M. A., Wang, Q., Wang, X., Cheng, Z., Liu, D. and Song, H. (2009). "Structural Basis for Translational Inhibition by the Tumor Suppressor Pdcd4." The EMBO Journal **28**: 274-285.
March, R. E. (1997). "An Introduction to Quadropole Ion Trap Mass Spectrometry." Journal of Mass Spectrometry **32**: 351-369.
Martin, J. W., Smithwick, M. M., Braune, B. M., Hoekstra, P. F., Muir, D. C. G. and Mabury, S. A. (2004). "Identification of Long-Chain Perfluorinated Acids in Biota from the Canadian Arctic." Environmental Science & Technology **38**: 373-380.

Mihailovich, M., Militti, C., Gabaldón, T. and Gebauer, F. (2010). "Eukaryotic Cold Shock Domain Proteins: Highly Versatile Regulators of Gene Expression." BioEssays **32**: 109-118.
Miller, W. L. (1988). "Molecular Biology of Steroid Hormone Synthesis." Endocrine reviews **9**(3): 295-318.
Miller, W. L. (2007). "Steroidogenic Acute Regulatory Protein (StAR), A Novel Mitochondrial Cholesterol Transporter." Biochimica et Biophysica Acta **1771**: 663-676.
Mollenhauer, M. A. M., Carter, B. J., Peden-Adams, M. M., Bossart, G. D. and Fair, P. A. (2009). "Gene Expression Changes in Bottlenose Dolphin, *Tursiops truncatus* Skin Cells Following Exposure to Methylmercury (MeHg) pr perfluorooctane Sulfonate (PFOS)." Aquatic Toxicology **91**: 10-18.
Mundt, D. J., Mundt, K. A., Luippold, R. S., Schmidt, M. D. and Farr, C. H. (2007). "Clinical Epidemiological Study of Empoyees Exposed to Surfactant Blend Containing Perfluorononanoic Acid." Occupational and Environmental Medicine **64**: 589-594.
Nicol, M. R., Papacleovoulou, G., Evans, D. B., Penning, T. M., Strachan, M. W. J., Advani, A., Johnson, S. J., Quinton, R. and Mason, J. I. (2009). Estrogen Biosynthesis in Human H295 Adrenocortical Carcinoma Cells. 13th Conference on the Adrenal Cortex, Burlingame, CA, Elsevier Ireland Ltd.
Noble, C. G. and Song, H. (2008). "Structural Studies of Elongation and Release Factors." Cellular and Molecular Life Sciences **65**: 1335-1346.
Nyhan, W. L. (2005). "Disorders of Purine and Pyrimidine Metabolism." Molecular Genetics and Metabolism **86**: 25-33.
Oakes, K. D., Sibley, P. K., Martin, J. W., MacLean, D. D., Solomon, K. R., Mabury, S. A. and Van Der Kraak, G. J. (2005). "Short-Term Exposures of Fish to Perfluorooctane Sulfonate: Acute Effects on Fatty Acyl-CoA Oxidase Activity, Oxidative Stress and Circulating Sex Steroids." Environmental Toxicology and Chemistry **24**(5): 1172-1181.
Olsen, G. W., Burris, J. M., Ehresman, D. J., Froehlich, J. W., Seacat, A. M., Butenhoff, J. L. and Zobel, L. R. (2007). "Half-Life of Serum Elimination of Perfluorooctanesulfonate, Perfluorohexanesulfonate, and Perfluorooctanoate in Retired Fluorochemical Production Workers." Environmental Health Perspectives **115**(9): 1298-1305.
Olsen, G. W., Burris, J. M., Mandel, J. H. and Zobel, L. R. (1999). "Serum Perfluorooctane Sulfonate and Hepatic and Lipid Clinical Chemistry Tests in Fluorochemical Production Employees." Journal of Occupational & Environmental Medicine **41**(9): 799-806.
Ortiz, P. A., Ulloque, R., Kihara, G. K., Zheng, H. and Kinzy, T. G. (2006). "Translation Elongation Factor 2 Anticodon Mimicry Domain Mutants Affect Fidelity and Diphteria Toxin Resistance." Journal of Biological Chemistry **28**(43): 32639-32648.
Oskarsson, A., Ullerås, E., Plant, K. E., Hinson, J. P. and Goldfarb, P. S. (2006). "Steroidogenic Gene Expression in H295R Cells and the Human Adrenal Gland: Adrenotoxic Effects of Lindane *in vitro*." Journal of Applied Toxicology **26**: 484-492.
Pappin, D. J. C., Hojrup, P. and Bleasby, A. J. (1993). "Rapid Identification of Proteins by Peptid-Mass Fingerprinting." Current Biology **3**(6): 327-332.
Porta, M., Puigdomènech, E., Ballester, F., Selva, J., Ribas-Fitó, N., Llop, S. and López, T. (2008). "Monitoring Concentrations of Persistent Organic Pollutants in the

General Population: The International Experience." Environment International **34**: 546-561.
Quinn, C. M., Wiles, A. P., El-Shanawany, T., Catchpole, I., Alnadaf, T., Ford, M. J., Gordon, S. and Greaves, D. R. (1999). "The Human Eularyotic Initiation Factor 4A1 Gene (EIF4A1) Contains Multiple Regulatory Elements That Direct High-Level Reporter Gene Expression in Mammalian Cell Lines." Genomics **62**: 468-476.
Rainey, W. E., Bird, I. M. and Mason, J. I. (1994). "The NCI-H295 Cell Line - A Pluripotent Model for Human Adrenocortical Studies." Molecular and Cellular Endocrinology **100**(1-2): 45-50.
Rainey, W. E., Bird, I. M., Sawetawan, C., Hanley, N. A., McCarthy, J. L., McGee, E. A., Wester, R. and Mason, J. I. (1993). "Regulation of Human Adrenal Carcinoma Cell (NCI-H295) Production of C19 Steroids." Journal of Clinical Endocrinology and Metabolism **77**(3): 731-737.
Rainey, W. E., Saner, K. and Schimmer, B. P. (2004). "Adrenocortical Cell Lines." Molecular and Cellular Endocrinology **228**(1-2): 23-38.
Ribes, D., Fuentes, S., Torrente, M., Colomina, M. T. and Domingo, J. L. (2010). "Combined Effects of Perfluorooctane Sulfonate (PFOS) and Maternal Restraint Stress on Hypothalamus Adrenal Axis (HPA) Function in the Offspring of Mice." Toxicology and Applied Pharmacology **243**: 13-18.
Roesli, C., Borgia, B., Schliemann, C., Gunthert, M., Wuderli-Allenspach, H., Giavazzi, R. and Neri, D. (2009). "Comparative Analaysis of the Membrane Proteome of Closely Related Metastatic and Nonmetastatic Tumor Cells." Cancer Research **69**(13): 5406-5414.
Rohrbough, J. G., Galgiani, J. N. and Wysocki, V. H. (2007). "The Aplication of Proteomic Techniques to Fungal Protein Identification and Quantification." Annals of the New York Academy of Science **1111**: 133-146.
Rosmond, R., Chagnon, Y. C., Holm, G., Chagnon, M., Pérusse, L., Lindell, K., Carlsson, B., Bouchard, C. and Björntorp, P. (2000). "A Glucocorticoid Receptor Gene Maker IS Associated with Abdominal Obesity, Leptin, and Dysregulation of the Hypothalamic-Pituitary-Adrenal Axis." Obesity Research **8**(3): 211-218.
Roth, U., Razawi, H., Hommer, J., Engelmann, K., Schwientek, T., Müller, S., Baldus, S. E., Patsos, G., Corfield, A. P., Paraskeva, C. and Hanisch, F. G. (2010). "Differential Expression Proteomics of Human Colorectal Cancer Based on a Synergeneic Cellular Model for the Progression of Adenoma to Carcinoma." Proteomics **10**(2): 194-202.
Seacat, A. M., Thomford, P. J., Hansen, K. J., Olsen, G. W., Case, M. T. and Butenhoff, J. L. (2002). "Subchronic Toxicity Studies on Perfluorooctanesulfonate Potassium Salt in Cynomolgus Monkeys." Toxicological Sciences **68**: 249-264.
Seth, R., Keeley, J., Abu-Ali, G., Crook, S., Jackson, D. and Ilyas, M. (2009). "The Putative Tumor Modifier Gne ATP5A1 is Not Mutated in Human Colorectal Cancer Cell Lines But Expression Levels Correlate with TP53 Mutations and Chromosomal Instability." Journal of Clinical Pathology **62**: 598-603.
Shi, X., Yeung, L. W. Y., Lam, P. K. S., Wu, R. S. S. and Zhou, B. (2009). "Protein Profiles in Zebrafish (*Danio Rerio*) Embryos Exposed to Perfluorooctane Sulfonate." Toxicological Sciences **110**(2): 334-340.
Shi, Z., Zhang, H., Ding, L., Feng, Y., Wang, J. and Dai, J. (2010). "Proteomic Analaysis for Testis of Rats Chronically Exposed to Perfluorododecanoic Acid." Toxicology Letters **192**: 179-188.

Shi, Z., Zhang, H., Liu, Y., Xu, M. and Dai, J. (2007). "Alterations in Gene Expression and Testosterone Synthesis in the Testes of Male Rats Exposed to Perfluorodecanoic Acid." Toxicological Sciences **98**(1): 206-215.
Skoog, K., Hauglid-Formo, G. and Økland, T. (2008). TA-2450/2008: Polybrominated Diphenyl Ethers and Perfluorinated Compounds in the Norwegian Environment. Oslo, Norwegian State Pollution Control Authority.
Slotkin, T. A., MacKillop, E. A., Melnick, R. L., Thayer, K. A. and Seidler, F. J. (2008). "Developmental Neurotoxicity of Perfluorinated Chemicals Modeled *in Vitro* " Environmental Health Perspectives **116**(6): 716-722.
Sohn, H. Y., Keller, M., Gloe, T., Morawietz, H., Rueckschloss, U. and Pohl, U. (2000). "The Small G-protein Rac Mediates Depolarization-induced Superoxid Formation in Human Endothelial Cells." The Journal of Biological Chemistry **275**(25): 18745-18750.
Staels, B., Hum, D. W. and Miller, W. L. (1993a). "Regulation of Steroidogenesis in NCI-H295 Cells - A Cellular-Model of the Human Fetal Adrenal." Molecular Endocrinology **7**(3): 423-433.
Staels, B., Hum, D. W. and Miller, W. L. (1993b). "Regulation of Steroidogenesis in NCI-H295 Cells: A Cellular Model of the Human Fetal Adrenal." Molecular Endocrinology **7**(3): 423-433.
Starkov, A. A. and Wallace, K. B. (2002). "Structural Determinant of Fluorochemical-Induced Mitochondrial Dysfunction." Toxicological Sciences **66**: 244-252.
Stevens, A. and Lowe, J. (2000). Pathology, Harcourt Publishers Limited.
Stigliano, A., Cerquetti, L., Borro, M., Gentile, G., Bucci, B., Misiti, S., Piergrossi, P., Brunetti, E., Simmaco, M. and Toscano, V. (2008). "Modulation of Proteomic Profile in H295R Adrenocortical Cell Line Induced by Mitotane." Endocrine-Related Cancer **15**(1351-0088/08/015-001): 1-10.
Stockwin, L. H., Bumke, M. A., Yu, S. X., Webb, S. P., Collins, J. R., Hollingshead, M. G. and Newton, D. L. (2007). "Proteomic Analysis Identifies Oxidative stress Induction by Adaphostin." Clinical Cancer Research **13**(12): 3667-3681.
Stomperudhaugen, E. S., Øverås, N. H. H., Langford, K., de Coen, W., Smolders, R. and Hylland, K. (2009). "Cellular Energy Allocation in *Hediste diversicolor* Exposed to Sediment Contaminants." Journal of Toxicology and Environmental Health, Part A **72**: 244-253.
Sugawara, T., Lin, D., Holt, J. A., Martin, K. O., Javitt, N. B., Miller, W. L. and Strauss, J. F. (1995). "Structure of the Human Steroidogenic Acute Regulatory Protein (StAR) Gene: StAR Stimulated Mitochondrial Cholesterol 27-Hydroxylase Activity." Biochemistry **34**: 12506-12512.
Tang, D., Kang, R., Zeh III, H. J. and Lotze, M. T. (2010). "High-Mobility Group Box 1 and Cancer." Biochimica et Biophysica Acta **1799**: 131-140.
Tateishi, K., Omata, M., Tanaka, K. and Chiba, T. (2001). "The NEDD8 System is Essential for Cell Cycle Progression and Morphogenetic Pathway in Mice." The journal of Cell Biology **155**(4): 571-579.
Thibodeaux, J. R., Hanson, R. G., Rogers, J. M., Grey, B. E., Barbee, B. D., Richards, J. H., Butenhoff, J. L., Stevenson, L. A. and Lau, C. (2003). "Exposure of Perfluorooctane Sulfonate during Pregnancy in Rat and Mouse. I: Maternal and Prenatal Evaluations." Toxicological Sciences **74**: 369-381.
Thomas, J. O. and Travers, A. A. (2001). "HMG1 and HMG2, and Related "Architectural" DNA-Binding Proteins." TRENDS in Biochemical Sciences **26**(3): 167-174.

Tomy, G. T., Budakowski, W. R., Halldorson, T., Helm, P. A., Stern, G. A., Friesen, K., Pepper, K., Tittlemier, S. A. and Fisk, A. T. (2004a). "Fluorinated Organic Compounds in an Eastern Arctic Marine Food Web." Environmental Science & Technology **38**(24): 6475-6481.

Tomy, G. T., Tittlemier, S. A., Palace, V. P., Budakowski, W. R., Braekevelt, E., Brinkworth, L. and Friesen, K. (2004b). "Biotransformation of N-Ethyl Perfluorooctanesulfonamid by Rainbow Trout (*Onchorhynchus mykiss*) Liver Microsomes." Environmental Science & Technology **38**(3): 758-762.

Tsubota, A., Matsumoto, K., Mogushi, K., Nariai, K., Namiki, Y., Hoshina, S., Hano, H., Tanaka, H., Saito, H. and Tada, N. (2010). "IQGAP1 and Vimentin Are Key Regulator Genes in Naturally Occuring Hepatotumorigenesis Induced by Oxidative Stress." Carcinogenesis **31**(3): 504-511.

Uwins, C., Deitrich, C., Argo, E., Stewart, E., Davidson, I. and Cash, P. (2006). "Growth-Induced Changes in the Proteome of *Heliobacter pylori*." Electrophoresis **27**: 1136-1146.

Villeneuve, D. L., Ankley, G. T., Makynen, E. A., Blake, L. S., Greene, K. J., Higley, E. B., Newsted, J. L., Giesy, J. P. and Hecker, M. (2007). "Comparison of Fathead Minnow Ovary Explant and H295R Cell-Based Steroidogenesis Assay for Identifying Endocrine-Active Chemicals." Ecotoxicology and Environmental Safety.

Wang, T., Wang, Y., Liao, C., Cai, Y. and Jiang, G. (2009). "Perspectives on the Inclusion of Perfluoroctane Sulfonate into the Stockholm Cencention on Persistent Organic Pollutants." Environmental Health Perspectives **43**(14): 5171-5175.

Whitehead, S. A. and Rice, S. (2006). "Endocrine-Disrupting Chemicals as Modulators of Sex Steroid Synthesis." Best Practice & Research Clinical Endocrinology & Metabolism **20**(1): 45-61.

Wu, L. L., Gao, H. W., Gao, N. Y., Chen, F. F. and Chen, L. (2009). "Interaction of Perfluorooctanois Acid with Human Serum Albumin." BMC Structural Biology **9**(31).

Wysocki, V. H., Resing, K. A., Zhang, Q. and Cheng, G. (2005). "Mass Spectrometry of Petides and Proteins." Methods **35**: 211-222.

Yamashita, N., Kannan, K., Taniyasu, S., Horii, Y., Petrick, G. and Gamo, T. (2005). "A Global Survey of Perfluorinated Acids in Oceans." Marine Pollution Bulletin **51**: 658-668.

Yeung, L. W. Y., Guruge, K. S., Yamanaka, N., Miyazaki, S. and Lam, P. K. S. (2007). "Differential Expression of Chicken Hepatic Genes Responsive to PFOA and PFOS." Toxicology **237**: 111-125.

Zhang, L., Pan, X. and Hershey, J. W. B. (2007). "Individual Overexpression of Five Subunits of Human Translation Initiation Factor eIF3 Promotes Malignant Transformation of Immortal Fibroblast Cells." Journal of Biological Chemistry **282**(8): 5790-5800.

Zhou, C., Arslan, F., Wee, S., Krishnan, S., Ivanov, A. R., Olivia, A., Leatherwood, J. and Wolf, D. A. (2005). "PCI Proteins eIF3e and eIF3m Define Distinct Translation Initiation Factor 3 Complexes." BMC Biology **3**(14).

Appendix 1: Equipments

Equipment/kits	
1,5 ml eppendorf tubes	VWR International AS, Oslo, Norway
7 cm Immobiline^{TM} DryStrips pH 3-10 NL	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
75 cm^2 flasks (T75)	BD Falcon, New Jersey, USA
96-well plates	VWR International AS, Oslo, Norway
Allprep® DNA/RNA/Protein Mini Kit	Qiagen, Crawley, UK
Blotting cassettes	MIDSCI, Saint Louis, Missouri, USA
Blotting filter papir	MIDSCI, Saint Louis, Missouri, USA
Bürker haematocytometer	Superior, Marienfeld, Germany
Cell strainer	BD Biosciences, NJ, USA
Cell-line H295R	American Type Culture Collection (ATCC # CRL-2128, ATCC, Manassas, VA, USA)
Coat-A-Count Cortisol kit	Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA
Coat-A-Count Estradiol kit	Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA
Coat-A-Count Total Testosterone kit	Siemens Medical Solutions Diagnostics, Los Angeles, CA, USA
Dry strip alligner	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
Dry strip cover fluid	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
Dulbecco's Modified Eagles Medium and Ham's F-12 Nutrient mixture (1:1)	Sigma D-2906; Sigma-Aldrich, Inc, St Louis, MO, USA
Falcon 10 ml	BD Falcon, New Jersey, USA
Falcon 50 ml	BD Falcon, New Jersey, USA
Foam pads	MIDSCI, Saint Louis, Missouri, USA
Immobiline IPG reswelling cassette	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
Immobilon-FL membrane	Milipore (UK) Ltd, Watford, UK
Invitrogen antioxidant	Invitrogen, Carlsbad, CA, USA
LiCor 2 colour marker	LI-COR Biosciences, Lincoln, NE, USA
Microcuvettes	VWR International AS, Oslo, Norway
Mini Protean 3 gel system	Bio-Rad Laboratories Ltd, Hemel Hempstead, UK
NuPage® Novex Antioxidant	Invitrogen, Carlsbad, CA, USA
NuPage® Novex Midi Bis-Tris Gel	Invitrogen, Carlsbad, CA, USA
NuPage® Novex Reducing Agent	Invitrogen, Carlsbad, CA, USA
Odyssey® Two-Color Protein Molecular Weight Marker	LI-COR Biosciences, Lincoln, NE, USA
Owl Bandit Transfer Tank	MIDSCI, Saint Louis, Missouri, USA
Pipette aid XP	Drummond Scientific co., PA, USA

Pipette PS Steril 1 ml	VWR International AS, Oslo, Norway
Pipette PS Steril 10 ml	VWR International AS, Oslo, Norway
Pipette PS Steril 2 ml	VWR International AS, Oslo, Norway
Pipette PS Steril 25 ml	VWR International AS, Oslo, Norway
Pipette PS Steril 5 ml	VWR International AS, Oslo, Norway
Pipette tips 1000p (filtered)	Fisher Scientific, Pittsburgh, PA, USA
Pipette tips 10p (filtered)	Fisher Scientific, Pittsburgh, PA, USA
Pipette tips 200p (filtered)	Fisher Scientific, Pittsburgh, PA, USA
Pipette tips 20p (filtered)	Fisher Scientific, Pittsburgh, PA, USA
Qia Shredder	Qiagen, Crawley, UK
RC DC Protein Assay	Bio-Rad Laboratories Ltd, Hemel Hempstead, UK
ReadyPrep™ 2-D Cleanup Kit	Bio-Rad Laboratories Ltd, Hemel Hempstead, UK
SeeBlue Plus 2 Pre-Stained Standard	Invitrogen, Carlsbad, CA, USA
Spectria Progesterone RIA kit	Orion Diagnostica, Espoo, Finland
Xcell SureLock Electrophoresis Unit	Invitrogen, Carlsbad, CA, USA
ZOOM® Gels	Invitrogen, Carlsbad, CA, USA

Chemicals	
0.25 % trypsin/0.53 nM EDTA	Gibco, Invitrogen, Paisley, USA
2-Mercaptoethanol	Sigma-Aldrich, Inc, Saint Louis, MO, USA
3-((3-Cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS)	Fisher Scientific, Pittsburgh, PA, USA
Acetonitrile	Rathburn Chemicals Ltd, Walkerburn, Scotland
Acrylamid	Sigma-Aldrich, Inc, Saint Louis, MO, USA
Alamar Blue	Invitrogen, Carlsbad, CA, USA
Ammounium persulphate	Sigma-Aldrich, Inc, Saint Louis, MO, USA
Ammounium sulphate	Sigma-Aldrich, Inc, Saint Louis, MO, USA
BD Nu-Serum	BD Bioscience, San Jose, CA, USA
Bovine serum albumin (BSA)	A7906, Sigma-Aldrich, Inc, Saint Louis, MO, USA
Bromophenol	Sigma-Aldrich, Inc, Saint Louis, MO, USA
Coomassie Blue G250	Sigma-Aldrich, Inc, Saint Louis, MO, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, Inc, Saint Louis, MO, USA
Dithiothreitol (DTT)	Sigma-Aldrich, Inc, Saint Louis, MO, USA
Dithiothreitol (DTT)	Fisher Scientific, Pittsburgh, PA, USA
Ethanol	Sigma-Aldrich, Inc, Saint Louis, MO, USA
Formic acid	Mallinckrodt Baker, Inc., Phillipsburg, NJ, USA
Glycerol	Sigma-Aldrich, Inc, Saint Louis, MO, USA
Glycine	Sigma-Aldrich, Inc, Saint Louis, MO, USA

Iodoacetamide	Sigma-Aldrich, Inc, Saint Louis, MO, USA
ITS+ Premix	BD Bioscience, San Jose, CA, USA
Methanol	Sigma-Aldrich, Inc, Saint Louis, MO, USA
N, N, N', N'-tetramethylenethylenediamine (TEMED)	Sigma-Aldrich, Inc, Saint Louis, MO, USA
N-Methylene Bis-acrylamid	Sigma-Aldrich, Inc, Saint Louis, MO, USA
Perfluorononanoic acid	Sigma-Aldrich, Inc, Saint Louis, MO, USA
Phosphatase Inhibitor Cocktail 1	Sigma-Aldrich, Inc, Saint Louis, MO, USA
Phosphatase Inhibitor Cocktail 2	Sigma-Aldrich, Inc, Saint Louis, MO, USA
Phosphate Buffered Saline (PBS) - ICN92810305	Fisher Scientific, Pittsburgh, PA, USA
Phosphoric acid	Sigma-Aldrich, Inc, Saint Louis, MO, USA
Protease Inhibitor Cocktail	Sigma-Aldrich, Inc, Saint Louis, MO, USA
Sodium dodecyl sulphate (SDS)	Sigma-Aldrich, Inc, Saint Louis, MO, USA
Tetrabutylammonium chloride	Sigma-Aldrich, Inc, Saint Louis, MO, USA
Tetrabutylammonium heptadecafluorooctanesulfonate	Sigma-Aldrich, Inc, Saint Louis, MO, USA
Thiourea	Fluka, Sigma-Aldrich, Sain Louis, Missouri, USA
Trizma-base	Sigma-Aldrich, Inc, Saint Louis, MO, USA
Trypsin	Promega UK, Southampton, UK
Tween	Sigma-Aldrich, Inc, Saint Louis, MO, USA
Urea	Fisher Scientific, Pittsburgh, PA, USA

Commercial buffers	
IPG equilibrating buffer	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
IPG Reswell buffer	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
MOPS 20x concentrate buffer	Invitrogen, Carlsbad, CA, USA
NuPage® 20x Transfer buffer	Invitrogen, Carlsbad, CA, USA
NuPage® Novex LDS Sample Buffer (4x)	Invitrogen, Carlsbad, CA, USA
NuPage® Novex MOPS running buffer	Invitrogen, Carlsbad, CA, USA
Odyssey Blocking Buffer	LI-COR Biosciences, Lincoln, NE, USA

Instruments	
Centrifuge, GS-6R Centrifuge	Beckman Coulter, Brea, CA 92822-8000 USA
CO2 incubator	Hera Cell 150, Heraeus, Hanau, Germany
EasyBreeze Gel Drier	Hoefer, Inc., Holliston, MA, USA
EPS 3000 XL Power Supply	Pharmacia Biotech
Flow-Cabinet, Kojair®	Tampere, Finland
Grant QBT2 Heatblock	Grant Instruments (Cambridge) Ltd., Herts,

	England
HCTultra PTM Discovery System	Bruker Daltonics Ltd., Coventry, UK
ImageScanner ^{TM} III	GE Healthcare, Uppsala, Sweden
Investigator ProGest robotic workstation	Genomic Solutions Ltd., Huntingdon, UK
Invitrogen Power Supply	Invitrogen, Carlsbad, CA, USA
LAF bench	ScanLaf AS, Lynge, Denmark
Microscope - Nikon TMS	Interfoto Instrument AS, Høvik, Norway
Multiphor II Electrophoresis System	GE Healthcare Bio-Sciences AB, Uppsala, Sweden
NanoDrop	Thermo-Scientific, Waltham, MA, USA
Odyssey infrared fluorescence imager	LI-COR UK Ltd. UK
Phoretic-1D Advanced software	Nonlinear Dynamics Ltd, Newcastle, UK
Power Pac 3000 Power Supply	Bio-Rad Laboratories Ltd, Hemel Hempstead, UK
SC110 Speedvac	Savant Instruments, Holbrook, NY, USA
UltiMate 3000 LC System	Dionex (UK) Ltd., Camberley, Surrey, UK
UV/VIS spectrophotometer	Lambda 1, Perkin-Elmer, Shelton, CT, USA
Vacusafe comfort	© INTEGRA Biosciences 2008, Chur, Switzerland
Victor 3™ 1420 Multilabel counter	Perkin Elmer, MA, USA
Vortex LR Labinco L24	Labinco, Breda, Netherlands
Wallac 1470 Wizard™ automatic gamma counter	Perkin Elmer, MA, USA
Waterbath, Lauda Ecoline 019	Lauda-Königshofen, Germany

Softwares	
Data Analysis software	Bruker Daltonics Ltd., Coventry, UK
Ingenuity Pathway Analysis	Ingenuity Systems Inc, CA, USA
JMP 8 software	SAS Institute Inc, Cary, NC, USA
LabScan software	GE Healthcare, Uppsala, Sweden
Phoretic-1D Advanced software	Nonlinear Dynamics Ltd, Newcastle, UK
Progenesis Samespots v 3.3	Nonlinear Dynamics Ltd, Newcastle, UK
R 2.10.1	The R Foundation for Statistical Computing

Appendix 2: Buffers used in protein isolation

Modified Reswell Buffer

Urea	2.01 g
Thiourea	0.7 g
CHAPS	0.2 g
DTT	0.015 g
MilliQ water	3 mL

Dissolve all the chemicals in water. Mix well, and make sure all the chemicals dissolves.

Appendix 3: Standards for protein quantification

Standards for Protein Assay:

Stock standard = 14,4 mg/ml

Standard 1 = 1.5 mg/ml: 70µl stock + 602µl dH2O

Standard 2 = 1.0 mg/ml: 200µl 1.5 mg/ml std + 100µl dH2O

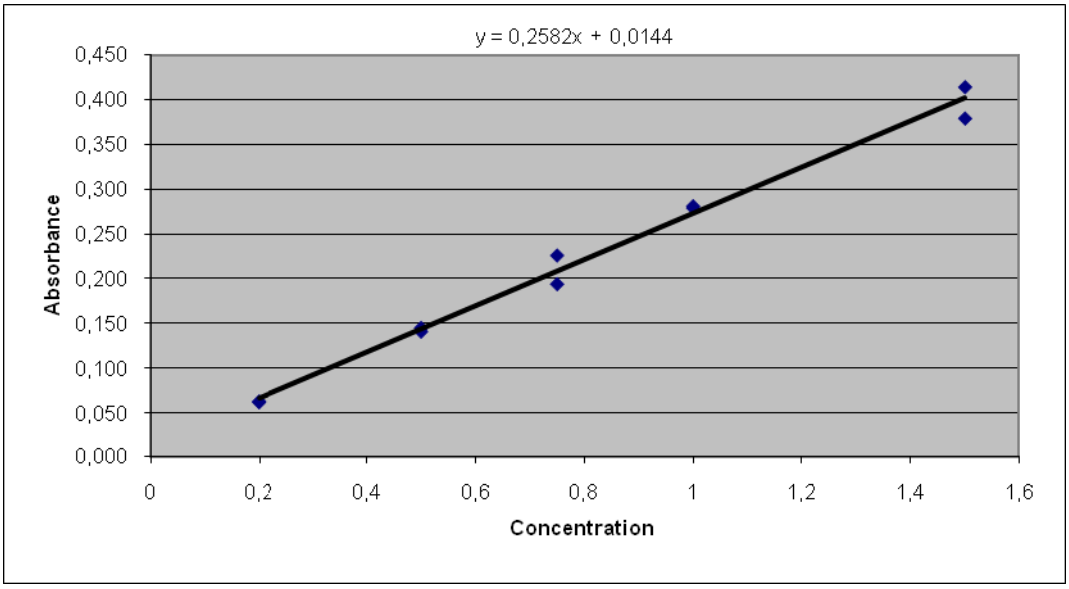
Standard 3 = 0.75 mg/ml: 100µl 1.5 mg/ml std + 100µl dH2O

Standard 4 = 0.5 mg/ml: 100µl 1.5 mg/ml std + 200µl dH2O

Standard 5 = 0.2 mg/ml: 20µl 1.5 mg/ml std + 130µl dH2O

Appendix 4: Standard curve and calculation after RC DC Protein assay

Prøve	Abs	Average	Stdev	% var	CV	Conc (µg/µl)	V (µl)	Ammount (µg)
Standard 1	0,414	0,397	0,025	9 %	6,242	1,5		
Standard 1	0,379					1,5		
Standard 2	0,279	0,280	0,001	1 %	0,505	1		
Standard 2	0,281					1		
Standard 3	0,194	0,210	0,023	15 %	10,775	0,75		
Standard 3	0,226					0,75		
Standard 4	0,145	0,143	0,003	3 %	1,978	0,5		
Standard 4	0,141					0,5		
Standard 5	0,063	0,063	0,001	2 %	1,131	0,2		
Standard 5	0,062					0,2		
Lav kontroll	0,098	0,105	0,010	13 %	9,428	0,35		
Lav kontroll	0,112							
Høy kontroll	0,393	0,378	0,021	8 %	5,612	1,41		
Høy kontroll	0,363							
0,1 % DMSO 020409	0,226	0,214	0,017	11 %	7,930	15,46	195	3014,87
0,1 % DMSO 020409	0,202							
150 µM TBA 020409	0,234	0,231	0,004	3 %	1,837	16,78	195	3271,65
150 µM TBA 020409	0,228							
175 µM TBA 020409	0,211	0,213	0,002	1 %	0,998	15,34	195	2992,22
175 µM TBA 020409	0,214							
200 µM TBA 020409	0,230	0,227	0,004	3 %	1,869	16,47	195	3211,23
200 µM TBA 020409	0,224							
150 µM PFOS 020409	0,135	0,143	0,011	11 %	7,443	9,92	195	1934,90
150 µM PFOS 020409	0,150							
175 µM PFOS 020409	0,028	0,034	0,008	33 %	23,218	1,48	195	288,50
175 µM PFOS 020409	0,039							
200 µM PFOS 020409	0,030	0,031	0,001	3 %	2,318	1,25	195	243,18
200 µM PFOS 020409	0,031							
175 µM PFNA 020409	0,153	0,144	0,013	13 %	8,839	10,04	195	1957,55
175 µM PFNA 020409	0,135							
200 µM PFNA 020409	0,138	0,144	0,008	8 %	5,420	10,00	195	1950,00
200 µM PFNA 020409	0,149							
0,1 % DMSO 030409	0,262	0,253	0,013	7 %	5,031	18,48	195	3603,95
0,1 % DMSO 030409	0,244							
150 µM PFNA 030409	0,231	0,227	0,006	4 %	2,492	16,47	195	3211,23
150 µM PFNA 030409	0,223							
175 µM PFNA 030409	0,301	0,287	0,021	10 %	7,157	21,08	195	4109,95
175 µM PFNA 030409	0,272							
200 µM PFNA 030409	0,229	0,225	0,006	4 %	2,835	16,27	195	3173,47
200 µM PFNA 030409	0,220							



The standard curve is made by plotting the absorbance of the standards against the known concentration. The formula is calculated, and used to calculate the protein concentrations in the samples.

Appendix 5: Buffers used in 1D-gels and 2D-gels

Phosphate Buffered saline (PBS):

Sodium chloride	12 g
Potassium chloride	0.3 g
DiSodium phosphate	1.73 g
Sodium dihydrogen phosphate	0.3 g

Adjust to a final volume of 1200 mL with MilliQ water. pH should be ca. 7.4 without adjustment.

IPG Reswell Buffer

Urea	2.01 g
Thiourea	0.7 g
CHAPS	0.2 g
DTT	0.015 g
MilliQ water	3 mL
IPG Buffer	50 µL

Add sufficient bromophenol to give a blue color to the solution.

30 % Acrylamid solution

Acrylamid	150 g
N-methylene Bis-acrylamid	4.0 g

Adjust to final volume of 500 mL with MilliQ water.

2M Tris-HCL, pH 8.8

Trizma-base	121.1 g

Dissolve in approximately 400 mL water, and adjust pH 8.8 with 6M HCL. Adjust to a final volume of 500 mL with MilliQ water.

0.5 M Tris-HCL, pH 6.8

Trizma-base	15.1 g

Dissolve in approximately 200 mL MilliQ water, and adjust pH 6.8 with 6M HCL. Adjust to a final volume of 250 mL with MilliQ water.

10 % Ammonium persulphate

Ammonium persulphate	1.5 g
MilliQ water	15 mL

Prepare fresh immediately before use

10 % Sodium Dodecyl Sulphate (SDS)

SDS	25 g

Adjust final volume of 250 mL with MilliQ water.

25 % Sodium Dodecyl Sulphate (SDS)

SDS	25 g

Adjust to a final volume of 100 mL with MilliQ water.

N, N, N', N'-tetramethylenthylenediamine (TEMED)

Use undiluted as obtained from the supplier.

3x Dissociation Buffer

0.5 M Tris- HCL, pH 6.8	2.0 mL
25 % SDS	1.7 mL
2-Mercaptoethanol	1.3 mL
Glycerol	2.0 mL

Add bromophenol blue to give a blue color to act as a tracking dye during electrophoresis.

Electrophoresis Running Buffer

Glycine	6.0 g
Trizma base	9.5 g
SDS	1.5 g

Adjust to a final volume of 1000 mL with MilliQ water.

Resolving gel composition

Stock solution	**Resolving gel concentration – 10 %**
30 % Acrylamid (mL)	6.67
2M Tris-HCL pH 8.8 (mL)	3.75
10 % SDS (mL)	0.2
MilliQ water (mL)	9.38
Catalysts	
TEMED (µL)	10
10 % (w/v) Ammonium persulphate (µL)	100

Stacking gel composition

Stock solution	**3.6 % stacking gel**
30 % Acrylamid (mL)	0.6
2M Tris-HCL pH 8.8 (mL)	1.2
10 % SDS (mL)	0.05
MilliQ water (mL)	3.14
Catalysts	
TEMED (µL)	5
10 % (w/v) Ammonium persulphate (µL)	50

IPG Equilibration Buffer

0.5 M Tris-HCL, pH 6.8	80 mL
Urea	288 g
Glycerol	240 mL
10 % SDS	160 mL

Adjust final volume to 800 mL with MilliQ water.

Gel fixing solution

Ethanol	500 mL
Phosphoric acid	20 mL

Adjust to a final volume of 100 mL with MilliQ water.

Colloidal Coomassie Brilliant Blue (CBB) Equilibration Buffer

Ammonium sulphate	34 g
Methanol	68 mL
Phosphoric acid	4 mL

Dissolve the ammonium sulphate in ~100 mL of water, and once it is in solution adjust to a final volume of 128 mL. Add the methanol and a white precipitate will form which dissolves after the addition of the phosphoric acid.

Appendix 6: Buffers used in LC-MS

Ammonium Bicarbonate (AMBIC) 100mM Solution

Dissolve 0.79 g Ammonium Bicarbonate in 100mL of UHQ water and filter (7.9mg/mL)

Dithiothreitol (DDT) 10mM solution

Dissolve 30mg DTT in 20mL of 50mM AMBIC and filter (1.5mg/mL)

Iodoacetamide 55mM solution

Dissolve 200mg Iodoacetamide in 20mL 50mM AMBIC and filter (10mg/mL)

Trypsin preparation

To a 20µg vial of porcine Trypsine add 1mL of UHQ water and mix thoroughly. Add to this 1µL of conc. Formic acid, which will help the longer-term storage of the trypsin solution as it's unlikely that whole vial will be required.

Dilute an aliquote of trypsin 10 times in 100mM AMBIC to give a working solution of 2ng per µL.

10 % Formic acid in water

Dissolve 10mL of conc. Formic acid in 90mL UHQ water.

Eluent A – 0.05 % Formic acid in water with 3 % acetonitrile

To 970mL of UHQ water add 30mL acetonitrile and 500µL formic acid

Eluent B – 80 % Acetonitrile in 0.04 % formic acid

To 800mL acetonitrile add 200mL UHQ water and 400µL formaic acid

Appendix 7: Buffers used in western blot

1x SDS Running buffer for outer chamber

50 mL of Invitrogen 20x NuPAGE MOPS Running Buffer

Fill up with dH2O to a final volume of 1L

1x running buffer for inner chamber

200 mL of 1x SDS Running buffer for outer chamber

500µL of Invitrogen NuPAGE Antioxidant

1x transfer buffer

200 mL of 20x Invitrogen NuPAGE transfer buffer

200 mL of 100 % methanol

2.0 g of SDS

2.0 mL of Invitrogen NuPAGEAntioxidant

1600 mL dH2O

PBS

Use 5 x ICN PBS tablets (cat no. 2810305) dissolved in 500mL dH2O

PBST

Use 500mL of PBS from above and add 1mL of Tween (Sigma P-2287)

Odyssey Blocking Buffer cat no. 927-40000